ALBUMIN MICROSPHERES FOR THE CONTROLLED RELEASE OF THERAPEUTIC AGENTS

BY

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To the memory of my Mother, whose love and devotion are still a source of guidance.

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Abstract of Dissertation Presented to the Graduate Council
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ALBUMIN MICROSPHERES FOR THE CONTROLLED RELEASE OF THERAPEUTIC AGENTS

By

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There is a growing interest in the use of human serum albumin microspheres (HSA/MS) as insoluble drug carriers for controlled delivery of therapeutic agents. HSA/MS synthesized by methods reported to date are somewhat hydrophobic and require surfactants for preparing injectable aqueous dispersions. Drugs must be incorporated during preparation and only moderate concentrations have been achieved (less than 15 wt %).

A new method for preparing hydrophilic, chemically cross-linked HSA/MS was developed in this study. Important aspects of this method include addition of the cross-linking reagent in the organic phase and use of concentrated polymer solutions as dispersion media. Uniform, round, solid, hydrophilic microspheres were readily prepared by this process.

media. Uniform, round, solid, hydrophilic microspheres were readily prepared by this process.

Surface properties were altered by chemical modification using amino acids, proteins, lectins or amino alcohols to quench residual aldehyde groups. Optical and scanning electron microscopy and electronic particle size characterization indicated that the process is versatile in producing solid MS in a wide size range (0.2 - 150 μ). HSA/MS prepared in this study are dispersed in aqueous media for injection, without the need for surfactants. Incorporation of synthetic polypeptides or modified dextrans into the HSA/MS was also achieved, thereby enabling modification of ionicity.

The hydrophilic HSA/MS reported here allow for the incorporation of therapeutic agents into the HSA/MS after synthesis. Although HSA/MS were readily prepared with up to 18 wt % adriamycin (AD), addition of polyglutamic acid (PGA) made possible the preparation of AD-PGA-HSA/MS containing up to 45 wt % of the AD through formation of the AD-PGA salt complex. HSA/MS containing other drugs were also prepared.

Drug release properties were measured in vitro. Different drug binding mechanisms (covalent, salt complexed and physical association) allowed for a wide range of release rates in both dynamic flow and static systems.

Drug-containing HSA/MS demonstrated reduced toxicity in vivo. HSA/MS injected into rabbit muscle remained immobilized for 2 to 6 weeks before biodegradation.

The results of this study indicate that HSA/MS have significant potential as carriers for intratumoral chemotherapy and other types of localized drug delivery.

1. INTRODUCTION

In the past, pharmacologists and pharmaceutical chemists have dealt with health problems primarily by the synthesis of new biologically active agents possessing improved efficacy. This is well demonstrated in the clinical treatment of cancer using chemotherapeutic agents. Since the birth of modern day cancer chemotherapy, a myriad of potentially useful drugs have been developed. However, although chemotherapy coupled with radiation and surgery has been effective with some forms of malignancies, solid tumors of the lung, breast and colon, representing over 60 percent of the cancer seen clinically today, have proven relatively resistant to conventional systemic chemotherapy.

The problem of poor efficacy for anti-tumor drugs is not due to their inability to kill cancer cells but rather their failure to adequately distinguish between normal and neoplastic tissue, thus subjecting the patient to serious systemic toxicity at high doses. Due to systemic drug distribution, the drug arrives at a tumor site at relatively low and often non-therapeutic concentrations. To obtain an effective drug concentration at a tumor site, the amount of drug in the blood would have to be maintained at very high levels; an impossibility because of the severe toxic burden to the host. There is therefore a need to localize drugs to maintain high concentrations at the tumor site and reduce systemic toxicity.

One method of drug localization studied in this laboratory is direct injection into tumor tissue. Even though low MW drugs tend to diffuse away from the target area at a rapid rate after intratumoral (i.t.) injections, encouraging therapeutic results have been obtained in animal studies. Experiments also have shown that use of multiple i.t. injections or drug-water/oil emulsions increases drug retention time with resulting higher cure rates in the animals. These studies point to a correlation between drug retention time and the effectiveness of i.t. chemotherapy.

A major objective of this research has therefore been the synthesis, characterization and evaluation of insoluble macromolecular drug carriers which might be immobilized by injection into solid tumors for controlling and localizing drug releasing properties. Although emphasis has been placed on cytotoxic drug delivery for cancer therapy, the treatment of bacterial and parasitic infections and arthritic inflammations has also been considered.

A carrier system was sought that would satisfy the following criteria: (1) ability to bind and release high concentrations of drugs, (2) stability after synthesis with clinically acceptable shelf life, (3) controllable particle size and dispersibility in aqueous media for easy injection, (4) drug release in active form over a controllable period of time, (5) biocompatability with suitable biodegradability, (6) easy chemical modification, and (7) controllable porosity or cross-linking.

One carrier system considered applicable is albumin in the form of microspheres. However, albumin microspheres produced by known vegetable oil emulsion methods did not meet many of these criteria. In particular, they are hydrophobic and high drug loadings are difficult to achieve.

A method was therefore sought to produce hydrophilic albumin microspheres that could be made with high concentrations of drugs. Hydrophilicity was regarded as important because water soluble drugs might then be adsorbed into the microspheres after synthesis. Additionally, wet chemistry for surface and bulk modification would be more readily accomplished. Such microspheres would also be more easily wetted and dispersed in a variety of aqueous media for injection without the need for surfactants.

This thesis concerns the development of a new method for preparing such hydrophilic albumin microspheres and drug-loaded compositions. The method developed is based on polymeric or steric stabilization. A detailed study of the effect of process variables was conducted. The physical and chemical properties of the albumin microspheres were characterized. Coulter counter techniques and scanning electron microscopy (SEM) were used to determine size distribution and morphology. Hydration was measured as a function of cross-linking density. Binding sites for chemical modification were determined using radioactive labeling.

Human serum albumin microspheres produced by this new procedure were used to bind cytotoxic drugs such as adriamycin (AD) and bleomycin (BLM). Compositions containing antibiotics such as gentimycin (GMC) and streptomycin (STM) were also prepared. Drug release properties were measured in vitro and some in vivo properties were studied. Incorporation of anionic polypeptides such as polyglutamic acid into the albumin was also investigated. Loading of basic drugs such as AD was increased in this way from 20 wt % to 40-50 wt %.

2. BACKGROUND

2.1 Conventional Chemotherapy

To date, cancer chemotherapy has primarily involved systematic methods of treatment, often after surgical removal of operable tumors, and radiation therapy (1). Although oncologists use this therapeutic approach for treating primary tumors as well as metastatic disease, it is unfortunate to note that cure rates for solid carcinomas have shown little improvement over the last 30 years (2).

The systemic distribution of anti-neoplastic agents in a patient, coupled with relatively non-specific cytotoxicity, has been the major stumbling block to successful clinical chemotherapy (3-6). Drug concentrations in tumor tissue usually are close to that in the blood with resulting non-specific toxicity and a low thereapeutic index (7-10).

2.2 Drug Targeting

The efficiency of systemic cancer chemotherapy would be vastly improved if anti-tumor drugs could be targeted specifically to neoplastic cells (11). Although there have been promising in vitro results, little success has been achieved in vivo (12).

One recently developed technique to increase the drug concentration at the tumor site that is used clinically today is intra-arterial or portal perfusion (13). This involves placing a catheter in the blood supply leading to the tumor. The drugs are then introduced through the catheter bathing the infected area. Although this treatment has shown some promise, drug retention time is still relatively short (14,15). To improve this technique, biodegradable microspheres have been infused either before or in combination with the oncolytic drugs to arrest the arterial blood flow and facilitate increased drug concentrations and longer local drug retention times (16-18). An obvious problem with these procedures is that the tumor area must have a well defined blood supply for catheterization.

2.2.1 Antibody Targeting

To optimize drug targeting, one should have a homing moeity that is able to seek out the targeted area carrying with it the therapeutic agent. This concept was first proposed in 1898 by Paul Ehrlich (19). It was not until half a century later that this proposal was implemented when several investigators published methods of attaching antibodies to cytotoxic drugs and demonstrated some in vitro specificity (20-22).

A general scheme involves the covalent attachment of anti-tumor drugs to antibodies that are specific for tumor associated antigens. Many such antigens have been reported (23). Theoretically, once the antibody-drug conjugate is administered, it should target the tumor mass, binding high concentrations of therapeutic payloads to the surface of the cancer cells. To retain active antibody-drug binding, only a few drug molecules may be bound to the antibody (24,25). However, low concentrations of bound drug will result in poor efficacy.

Rowland et al. (26) overcame this problem by attaching the antibody to a suitable carrier such as functionalized dextran, or polyglutamic acid. The carrier was first used to bind high concentrations of drug. This allowed retention of antibody activity (27). A serious problem that has yet to be resolved is the preparation and binding of suitable antibodies that are highly specific for tumor cells in vivo. With the development of monoclonal antibodies, there was speculation that this major stumbling block would soon be removed. Unfortunately, monoclonal antibodies have not yet shown improved efficacy for tumor targeting in vivo as compared with polycolonal antibodies (28).

2.2.2 Macromolecular Drug Targeting

Macromolecular drug conjugates administered by i.v. or intraperitoneum (i.p.) routes in animals have demonstrated reduced toxicity and preferential tumor uptake as compared to free drug, even though the large molecules do not possess obvious specificities or drug targeting characteristics

(29,30). One observation is that tumor cells display a higher endocytic uptake of the macromolecules by tumor cells (31). To exploit this increased phagocytosis of some cancer cells, a variety of carriers have been investigated, such as albumin, polylysine, polyglutamic acid, dextran, liposomes, artificial cells, erythrocytes and polypeptide microspheres. Each of these carrier systems has its own advantages and disadvantages. Extensive reviews have been published (32-34).

The need for drug dissociation from the carrier to become active, or the expression of cytotoxicity while still covalently bound to a macromolecule, is still an uncertain mechanistic issue. Ryser amd Shem (35) showed that when methotrexate was bound to polylysine there was a drug uptake in Chinese hamster ovary cells which are normally resistant to methotrexate transport across the cell membrane. The conjugate inhibited the growth of these cells whereas the free drug did not. Ryser concluded that the methotrexate is "piggybacked" into the cells where the inhibitory effect of the conjugate is due to intracellular hydrolysis of the polymer thereby releasing the methotrexate. This view is shared by Trouet et al. (36). They demonstrated that by complexing adriamycin with DNA, the in vitro bacteriostatic effect usually seen for adriamycin alone was eliminated. When a lysosomal enzyme extract was added to the DNA drug complex, bacteriostatic activity was again observed. This suggested that the enzyme released the active drug. These observations have led to the hypothesis that the conjugate enters the cell by endocytosis, and the complex is degraded intercellularly releasing the active drug.

However, it has been recently demonstrated that adriamycin (AD) covalently bound to a non-biodegradable microsphere retained full cytotoxic activity when tested against human leukemia in vitro (37). It was also shown that the AD remained bound to the microspheres during all phases of cell growth and death. Cytotoxic activity was thought to be enacted by membrane disruption. Terry (38) also has demonstrated that AD, attached to soluble poly-glutaraldehyde, was still able to bind DNA in vitro. Thus, drugs modified with macromolecules may exhibit conventional mechanisms for cytotoxic activity as well as new cytotoxic pathways.

Further research is still required in this area to determine if different anti-neoplastic agents retain their activity when covalently attached to large macromolecule carriers. This mechanistic understanding is of vital importance for the design of new drug conjugates.

2.3 Intratumor Chemotherapy

A physical approach to drug targeting is intratumor (i.t.) chemotherapy, the direct injection of therapeutic agents into solid tumors. This has been one of the few drug targeting methods that has shown significant success for the complete cure of some animal tumors (39-43). The technique has the advantage of providing high initial concentrations of drug at the tumor site with reduced systemic toxicity. Scott (39,40) demonstrated that the i.t. injection of Corynebacterium parvum (C. parvum), an immunostimulant, caused the complete regression of solid tumors in mice, but i.v. injections had no effect. Similar results were obtained by Likkite and Halpern (41).

With chemotherapy, a solid ascitic hepatocarcinoma in rats was cured by i.t. injection of bleomyein (BLM) (42). No cures occurred when the drug was given i.v. McLaughlin et al. (43) demonstrated that cytotoxic drugs such as AD and mitomycin (MC) also cure solid tumors in the line-10 guinea pig hepatoma. It should be noted that in these animal studies, not only was the primary tumor cured but untreated metastatic foci also regressed. It was also shown that in most cases, cured animals exhibited specific resistance to new tumor challenges, indicating a specific immune response. These encouraging results were the basis of our further involvement in the study of i.t. chemotherapy. In human studies by Klein (44) on local chemotherapy culminated in the development of a topical 5-fluorouricil treatment of some skin cancers. One of the few successful drug targeting techniques used clinically today involves the use of i.t. injections of Bacillus Calmette-Guerin (BCG) in malignant melonoma (45).

Unfortunately, there is a wide-spread belief among oncologists that inserting a hypodermic needle into a tumor will promote metastasis. This belief should be dispelled as the use of fine needle byopsies becomes increasingly common for cancer diagnosis. This question of provoking metastasis coupled with other potential problems associated with i.t. chemotherapy (i.e. necrotic lesions at the injection site and systemic spread of drugs away from the tumor area) has substantially suppressed the use of i.t. chemotherapy for human use.

The systemic spread of drugs from a local i.t. injection has been studied. Drug concentration was measured as a function of time following i.t. injection of BLM into solid tumors in rats (42). Within minutes after the injection, over 50% of the drug had left the tumor area (Figure 1). When the BLM was emulsified with oil, drug retention time increased, inducing a higher cure rate in the rat hepatocarcinoma model (Table 1). These results were confirmed by McLaughlin et al. (46) using AD in the guinea pig hepatoma. Also, dividing the dose of one i.t. injection of AD into three injections spaced 1 to 2 hours apart increased the efficacy in the line-10 guinea pig hepatoma (Goldberg, unpublished data). Drug retention time in the tumor was deemed an important factor for increased cure rates.

To circumvent the problems of i.t. injection, we have been involved in the development of soluble drug carriers with tissue fixing properties (38) and insoluble drug conjugates that would remain physically immobilized. These systems would have drug releasing properties for longer drug

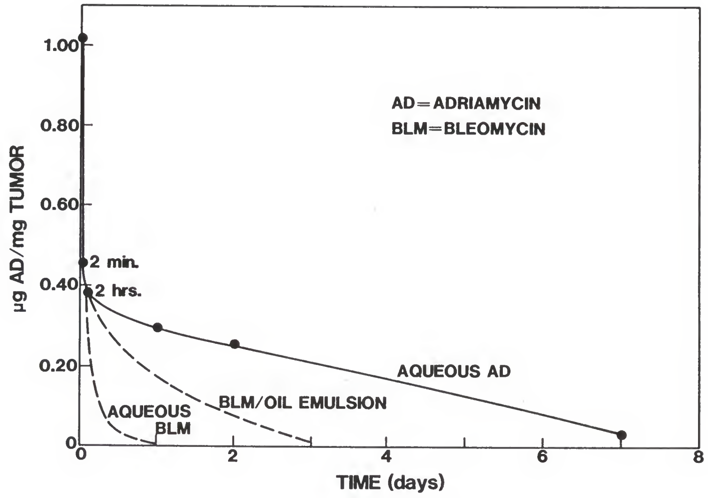


Figure 1. Concentration of AD and BLM in Tumor Tissue vs Time of Removal.

Table 1

Intratumor BLM Injection

	Time (mins) after Injection				
BLM Administered by	10	30	60	180	Rat Recovery
Sesame Oil Emulsion i.t.	8	3	2	0*	15/20**
Aqueous Solution i.t.	6	0	-	-	8/20
Aqueous Solution i.v.	0	-	-	-	0/20

*(μg/g) of BLM in tumor vs time
**Complete regression of tumor

retention in the tumor while reducing necrosis and systemic spread of the drugs.

2.4 Controlled Release of Therapeutic Agents

A drug delivery system that has been tested extensively in vivo with some success is the surgical implantation of a controlled release device. This may consist of either a diffusion membrane surrounding a drug core or a solid matrix that is permeable to the dispersed therapeutic agent (47). These devices might be utilized by either (1) implants near the target site to release the drug in high local concentrations, or (2) implants for systemic controlled release (48).

Interest in this type of drug delivery was stimulated in 1963 by Folkman and Long (49). While performing in vitro studies on artificial heart valves composed of silicone rubber, they observed that the valve had the ability to adsorb certain lipid soluble dyes from solution and subsequently release these dyes. This observation led to the development of silicone rubber capsules containing a cardioregulatory drug (digitoxin), these acted as a drug depot after surgical implantation. Other therapeutic agents were also used in this manner (50). Drug release is by a diffusional process through the silicone. Solubility parameters determined the rate of release (51). Alternate membrane polymers were studied, such as polyethylene, nylon, polycarbonate, Teflon and Cellophane, but were found to be inferior for drug release as compared to the silicone rubber (52). The size of the silicone capsules required surgical insertion, an undesirable method of administration for some diseases such as cancer.

Microcapsules are another type of controlled release system. They consist of a drug core encased in an ultrathin membrane usually formed from an insoluble polymer. Their small size allows for injection, so they can be easily introduced into the body by the parenteral route. Drug release may be mediated by various mechanisms including diffusion, erosion and rupture (53). Early microcapsule formulations used synthetic polymers such as nylon, polyvinylalochol, polyurethane and polyphenylesters (54,55). These materials were somewhat biocompatible but their nonbiodegradability restricts their use, since the depleted capsule must be surgically removed. This problem was overcome by the use of biodegradable polymers, such as polylactic acid (56) and polycaprolactone (57), which have been used successfully in the synthesis of microcapsules and microspheres. However, the difficulty of synthesis coupled with low drug loadings made these materials of doubtful value for our studies.

2.5 Albumin Microspheres for Localized Chemotherapy

Human serum albumin (HSA) is a blood plasma protein that has been recognized as a natural circulatory drug carrier (58,59). Equilibrium binding to various therapeutic agents depends primarily on hydrophobic and electrostatic interactions (60). This type of drug binding avoids the need for covalent attachment between drug and carrier and may facilitate drug release. HSA is also degraded in vivo.

These unique properties of HSA have led to the development of HSA/microspheres (MS) as drug carriers and studies by a number of investigators (61-66). The biodegradability of HSA/MS is also important and is a function of the degree of albumin cross-linking, porosity, and accessibility of microspheres to enzymatic and phagocytic process in the body (67).

Targeting of HSA/MS has been studied with magnetically responsive microspheres (68,69). Using magnetic HSA/MS with incorporated AD, Widder et al. (70) was able to obtain complete regressions with 80% cures in non-metastatic solid sarcomas located on the upper tail segments of rats. Targeting was accomplished by placing a magnetic field around the tumor mass, and infusing the AD-HSA/MS into the blood stream below the tumor area. High concentrations of drug were found at the tumor site with negligible amounts in other tissue. However, Sugibayashi et al. (71), employing the same technique for lung metastasis in rats (a deeper tissue model), showed only a marginal increase in AD concentration in the lungs with no regressions of the solid tumors. Because the tumor site must be in an accessible area for application of an external magnetic field, this technique is impractical for large areas of the body. Another potential dilemma is the elimination of the ferromagnetic particles used in the magnetic HSA/MS. Problems might arise from deposition in the reticuloendothelium system with subsequent formation of emboli.

We became interested in HSA/MS because they seemed to be uniquely suitable for i.t. injection. However, it was considered important to improve upon current methods for synthesis because of the hydrophobicity and relative low concentration of drug incorporation possible with previous methods for producing HSA/MS (61-64,72-74). These methods are basically modifications of the process developed in 1969 by Rhodes et al. (75) involving either denaturation at elevated temperatures (110-165) or chemical cross-linking in vegetable oil or isooctane emulsions (73,76). Because small amounts of surfactants are needed to disperse such microspheres in water, they appear to be somewhat hydrophobic because of the method of formation. Widder et al. (77) hypothesized that hydrophobicity is due to the polar regions of the albumin aligning at the oil-water interface to form a hydrophobic crust or mantle at room temperature. Since their process involves thermal denaturation for microsphere stabilization, a further increase in surface hydrophobicity may occur due to additional albumin conformational changes and surface binding of oil at elevated temperatures. Surface hydrophilicity is important because (1) a hydrophilic albumin surface may enhance surface physical and chemical behavior in vivo, (2) surfactants presently used to disperse HSA/MS may influence tissue interactions, drug release and activity, (3) a hydrophilic surface enables additional wet chemistry to be performed for surface modifications, and (4) high concentrations of water soluble drugs may be incorporated after synthesis of microspheres, an impossibility for hydrophobic HSA/MS.

Many investigators have explored the use of these hydrophobic microspheres in cancer chemotherapy with emphasis on AD, 5-fluorouricil and mercaptopurine (8,9,72,74). The therapeutic agents are incorporated into the microspheres before formation exposing the sensitive cancer drugs to degradative conditions. The vegetable oil denaturation technique achieves only low drug loadings (less than 15 percent) and degrades chemically and thermally sensitive drugs.

The clinical importance of cancer therapy has resulted in major emphasis on cancer drug targeting by most investigators. Treatment of other diseases with drug-HSA/MS has been generally overlooked but may prove important. In addition to HSA/MS for cancer therapy, this study has therefore also examined other therapeutic agents in albumin microspheres. These drugs include antibiotics for bacterial infections, such as gentamycin (GMC), streptomycin (STM) and steroids for inflammation diseases.

One of the most common bacterial pathogens is Pseudomonas aeruginosa. Pseudomonas is probably the most important cause of bacterial infections of the cornea. Keratitis produced by pseudomonas is difficult to treat by conventional instillation of antibiotic eye drops (78). Aminoglycosides such as gentamycin and tobramycin have been the most effective antibiotics used in the last decade for cornea infections (79). However, the delivery of adequate concentrations of these drugs to corneal ulcers caused by pseudomonas remains a serious practical problem for opthalmologists. Frequent installation of drops or applications of ointment often requires hospitalization for around the clock treatment (80). HSA/MS containing GMC may have value for prolonging localized controlled release of GMC in treatment of eye infections. GMC-HSA/MS were therefore synthesized and some properties were determined as part of this study.

A major parasitic infection in cattle has been studied as a potential candidate for HSA/MS therapy. This is Brucillosis Abortus. The infection localizes in the pregnant uterus (frequently causing abortion) as well as in the mammary gland. Healthy animals may shed brucellae in their milk for years. This disease can also infect humans (81).

The loss suffered by the cattle industry to this disease is enormous. Diagnosis results in destruction of the animals since there is no effective treatment. Streptomycin is the drug of choice for treatment. However, since brucillosis reside inside macrophages, they are protected from the antibiotic. Recent studies in this laboratory have demonstrated preferential uptake of hydrophilic HSA/MS by macrophages. Theoretically, once macrophages have injested HSA/MS containing streptomycin, the antibiotic would be released inside the cell thereby killing the brucella. STM-BSA/MS were therefore synthesized and some properties determined as part of this study.

Another application of HSA/MS for local chemotherapy considered is the treatment of rheumatoid arthritis. This crippling disease affects a large number of people in the United States each year (82). Intra-articular injections of corticosteroids are a valuable adjunct to the treatment of rheumatoid arthritis. However, this type of therapy must use large concentrations of the steroid because there is a very short retention time in the joint area. One approach to increase steroid retention time that has been studied is the use of liposomes containing corticosteroids. This type of treatment has shown some increased efficacy in patients (83). However, due to the numerous problems associated with liposomes as drug carriers, HSA/MS may be a better alternative and will be studied in future work.

We have therefore initiated preliminary studies with the synthesis of polylysine/MS. The cationic charge of these MS may inable the binding of corticosteroids in high concentrations so that drug uptake and release may be studied.

3. MATERIALS AND METHODS

3.1 Synthesis of Hydrophilic Human Serum Albumin/Microspheres: Unquenched (U) and Cross-Linked with Glutaraldehyde

3.1.1 In Polymethylmethacrylate-Chloroform/Toluene Dispersion

HSA (0.150 g) (Sigma, recrystallized and lyophilized) was dissolved in 0.5 ml water in a 16 x 125 mm test tube (this size test tube was used throughout all the following procedures except where noted). This solution was added drop wise to a 25 wt % solution of polymethylmethacrylate (PMMA) (Polyscience, intrinsic viscosity 1.4) in a mixture of 1.5 ml chloroform and 1.5 ml toluene in a screw cap test tube. The mixture was dispersed with a vortex mixer (Vortex Genie Scientific Industries, Inc.) for two minutes at a power setting of nine. Glutaraldehyde was used to cross-link the albumin. Aqueous glutaraldehyde 1.0 ml (25 wt %) (Polyscience) and 1.0 ml of toluene were combined in a 13 x 100 mm test tube. The two phases were dispersed by ultrasonification (Heat Systems-Ultrasonics, Model W-374) with a microtip power head attachment (20 secs at 50 watts). The resulting toluene solution of glutaraldehyde (0.14 mmoles) was allowed to phase separate, pipeted off, and combined with the albumin dispersion. After addition of the glutaraldehyde saturated toluene, the albumin dispersion was mixed with a rotary mixer (Labquake Labindustries) at room temperature (r.t.) for 8 hrs. The resulting cross-linked HSA/MS-U were washed to remove all PMMA dispersant by the addition of 10.0 ml of acetone, the test tube briefly agitated and then centrifuged (2000 RPM x 2 min), the supernate was discarded and the HSA/MS-U pellet was resuspended with an additional 10.0 ml of acetone. This wash procedure was repeated eight times. After the last wash, HSA/MS-U were allowed to air dry. The product was a brown powder, 0.122 g, 81% yield. The average diameter of the HSA/MS was 29 μm as determined by optical microscopy (Nikon Bihot).

3.1.2. In Polyoxyethylene/Polyoxypropylene Copolymer (Poloxamer 188) - Chloroform Dispersion

HSA (0.150 g) was dissolved in 0.5 ml of water in a test tube. This solution was added dropwise to a 25 wt % solution of Poloxmer 188 (BASF Wandotte Corp., MW 8430) in 4.0 ml chloroform in a screw cap test tube. The mixture was dispersed with a vortex mixer for two minutes at power setting nine. Glutaraldehyde was used for cross-linking and was prepared with chloroform by sonication as previously described in procedure 3.1.1. After addition of the glutaraldehyde saturated chloroform, the albumin dispersion was mixed with a rotary mixer at r.t. for 6 hrs. The resulting cross-linked HSA/MS-U were washed to remove all

Poloxmer 188 dispersant as described in procedure 3.1.1. The product was a brown powder, 0.115 g, 77% yield. The average diameter of HSA/MS was 20-40 µm as determined by optical microscopy.

3.1.3 In Cellulose Acetate Butyrate-Ethylene Dichloride Dispersion

HSA (0.150 g) was dissolved in 0.5 ml of water in a test tube. This solution was added dropwise to a 3.0 wt % solution of CAB (Polyscience, MW 73,000) in 4.0 ml of ethylene dichloride in a screw cap test tube. The mixture was dispersed with a vortex mixer for two minutes at power setting nine. Glutaraldehyde was used for cross-linking and was prepared as described in procedure 3.1.1. After addition of the glutaraldehyde saturated toluene, the dispersion was mixed with a rotary mixer at r.t. for 24 hrs. The resulting cross-linked HSA/MS were washed to remove all CAB dispersant and dehydrated as described in procedure 3.1.1. ✓ The product was a brown powder, 0.110 g, 73% yield. The average diameter of the HSA/MS-U was 25 µm determined by optical microscopy.

3.1.4 In Polycarbonate-Chloroform Dispersion

HSA (0.156 g) was dissolved in 0.5 ml of water in a test tube. This solution was added dropwise to a 20 wt % solution of polycarbonate (General Electric, MW 32,000) in

4.0 ml of chloroform. Glutaraldehyde was used for cross-linking and was prepared as described in procedure 3.1.2. After addition of the glutaraldehyde saturated chloroform, the mixture was mixed with a rotary mixer at r.t. for 16 hrs. The resulting cross-linked HSA/MS were washed to remove all polycarbonate dispersant by the addition of chloroform (8 X, 10.0 ml volumes), then acetone (8 X, 10.0 ml volumes) and water (4 X, 5.0 ml volumes). After the water wash, MS were examined with an optical microscope and stored frozen at 0°C. Average diameter of the MS was between 10-50 μm. Yield was not measured.

3.2 Various Cross-Linking Reagents for Microsphere Stabilization

3.2.1 Bovine Serum Albumin/Microspheres Unquenched Cross-Linked with Tolylene 2,4-Diisocyanate (TDC)

BSA (0.161 g) was dissolved in 0.5 ml of water in a test tube. This solution was dispersed in the PMMA solution as described in procedure 3.1.1. TDC (Aldrich) was used to cross-link the albumin. Aqueous TDC (80 percent 0.46 mmoles) 1.0 ml, and 1.0 ml of toluene were combined in a 2.0 ml volumetric flask. After mixing well, 1.0 ml of the TDC/toluene solution was added to the albumin dispersion. The dispersion was then mixed with a rotary mixer at r.t. for 21 hrs. BSA/MS-U were washed to remove all PMMA dispersant by centrifugation with acetone then water. After the water wash, the white pellet was frozen in liquid nitrogen and lyophilized yielding 0.246 g of a dry, free flowing white powder.

3.2.2 Bovine Serum Albumin/Microspheres Unquenched Cross-Linked with 1,6-Diisocyanatohexane (DCH)

BSA (0.163 g) was dissolved in 0.5 ml of water in a test tube. The solution was dispersed in the PMMA solution as described in procedure 3.1.1. Aqueous DCH (98 percent 5.8 mmoles, Aldrich) 1.0 ml, and 1.0 ml toluene were combined in a 2.0 ml volumetric flask. After mixing well, 1.0 ml of the resulting solution was added to the albumin dispersion and mixed at r.t. for 21 hrs. BSA/MS-U were washed and dehydrated according to the procedure in 3.3.1. The product was a white powder, 0.123 g, 76% yield.

3.2.3 Bovine Serum Albumin/Microspheres Cross-Linked with Aldehyde-Sucrose

Synthesis of the aldehyde-sucrose was by a modification of a dextran penodate oxidation reaction. Sucrose (3.423 g, 0.010 moles) dissolved in 50.0 ml of water was combined with sodium periodate (6.420 g, 0.046 moles) dissolved in 50.0 ml of water in a 250 ml erlenmeyer flask. The 100 ml solution was rigorously purged with nitrogen, covered with aluminum foil to protect from light and stirred magnetically for 16 hrs at room temperature. To the solution, excess silver nitrate was added to precipitate $NAI0_3$ and $NaI0_4$; the insoluble material was removed by centrifugation in two 50 ml polypropylene centrifuge tubes. The supernate was frozen in liquid nitrogen and lyophilized. The product was a white powder, 8.235 g. Excess silver nitrate and any residual $NaIO_3$, $NaIO_4$ was removed with a two phase ion exchange column. The column was 30 cm x 4 cm I.D., with a head pressure of 5.0 cm; the mobile phase consisted of water (pH 7.0) and the flow rate was 2.0 ml/minute. The bottom half of the column was packed with an anionic exchange resin (15 cm) (Amberlite IR-120). The starting aldehyde-sucrose material (2.530 g) was dissolved in 2.0 ml water; once loaded onto the column, 50.0 ml of the mobile phase was collected, frozen and lyophilized, producing 1.580 g, 46% yield of a white crystalline powder. Standard KBr pellets were made of the modified sucrose for FTIR (Nicolet MX-1) to confirm the presence of aldehyde functional groups.

BSA (0.150 g) was dissolved in 0.5 ml of water in a test tube. The protein solution was dispersed in a PMMA mixture as described in procedure 3.1.1. Aldehydic sucrose was used to cross-link the albumin molecules, 0.600 g; 1.75 mmoles of the crystalline white material was dissolved with 1.0 ml of water in a test tube; to this 1.0 ml of toluene was added and the two phase solution dispersed by ultrasonification as described in procedure 3.1.1. After addition of the aldehydic sucrose saturated toluene, the dispersion was mixed with a rotary mixer at r.t. for 25 hrs. The stabilized MS were washed out of the polymer dispersant and dehydrated as previously described in procedure 3.1.1. The product was a brown powder, 0.089 g, 59% yield. Average diameter of the microspheres was between 20-40 μm as determined with optical microscopy.

3.3 Microscopic Characterization of Microspheres

Size and morphology were characterized by scanning electron microscopy (SEM) (Jeol Model 35C) at either 20 or 25 kilovolts. SEM samples were prepared by applying a 200 angstrom coating of gold-palladium on samples using a sputter coater (Hummer Model V) in an argon atmosphere with a digital thickness monitor. HSA/MS were synthesized as described in procedure 3.1.1 and had an average diameter of 30 μm. A sample of the dry MS (0.010 g) was added to 2.0 ml of water and 0.5 ml of a protein dye (Coumassie blue G-250, BiotRad Laboratories) in a test tube. This solution was allowed to react for 1.0 hrs at room temperature. After addition of 3.0 ml of water, the test tube was briefly shaken and then centrifuged, the supernate was discarded and the blue pellet was resuspended in 5.0 ml of water and examined by optical microspcopy.

3.4 Effect of Polymer Dispersant Concentration, PMMA and Polxamer Concentration Gradients

HSA/MS were prepared as described in procedures 3.1.1 and 3.1.2. The concentrations of the polymer solutions were varied from 0 to 25% in 2% increments. After addition of the glutaraldehyde saturated toluene, the dispersions were mixed with a rotary mixer at r.t. for 16 hrs. After cross-linking the dispersions were evaluated for HSA/MS-U stability against aggregation; see Table 2.

3.5 Size Distributions

3.5.1 Measurement of Microsphere Size Distribution as a Function of Dispersion Time

HSA/MS were prepared as described in procedures 3.1.1. and 3.1.2. Dispersion times of 1, 3, 5 and 8 minutes were used at constant energy input (speeding setting 9 on vortex mixer). The washed MS were suspended in 10.0 ml of water. Aliquots were withdrawn from a well-shaken sample and placed on a SEM sample holder. One hundred random HSA/MS for each dispersion time were counted and measured. Aliquots (10 µliter) were also added to 150.0 ml of a continuously stirred 0.14 M potassium chloride solution. A 1.0 ml sample was withdrawn over a 4-sec time span by an electronic particle size counter (Coulter Counter Model TA11) equipped with a 100-micron sampling aperature. The counter was calibrated with 9.76 µm polystyrene microparticles. This enabled accurate size measurements from 2 to 40 µm. The total number of HSA/MS-U counted ranged from 7000 to 23,000 per sample; see Table 3.

3.5.2 Measurement of Microsphere Size Distributions as a Function of Energy Input

HSA/MS were prepared as described in procedure 3.1.1 using PMMA as the dispersant. Time was held constant

Table 2

Effect of Polymer (PMMA) Dispersant Concentration for HSA/MS-U

WT % PMMA	Dispersion Time (min)	Dispersion Stability*
0 - 8	1	-
	5	-
10	1	+
	5	-
12	1	+
	5	-
14	1	+
	5	-
16	1	+
	5	-
18	1	+
	5	-
20	1	+
	5	-
25	1	+
	5	+
25 - 30	1	+
	5	+

* - = Aggragation during cross-linking
+ = Stabilization during cross-linking

Table 3

HSA/MS-U Size Distribution vs Dispersion Time
at Constant Energy (Setting 9)

Dispersant* Medium	Dispersion Time (mins)	Mean diameter, μm SEM	Mean diameter, μm Coulter Counter
A	1	22	19
B		12	11
A	3	12	12
B		10	10
A	5	10	11
B		8.5	9
A	8	8.5	9
B		7	7

Note: Date plotted in Figure 30 and Figure 31.

*A, denotes PMMA as polymer dispersant.
B, denotes Poloxamer 188 as polymer dispersant.

(10 min) and energy input was varied by adjusting the speed setting on the vortex mixer, using speeds of 2, 4, 6 and 8. The washed HSA/MS were suspended in 10.0 ml of distilled water and size distributions were measured by the SEM technique, Table 4.

3.6 Synthesis of Sub-Micron Microspheres

HSA samples (1) 0.150 g, (2) 0.154 g, and (3) 0.164 g were dissolved in 0.5 ml of water in test tubes. These solutions were added dropwise to 3.0 percent solutions of

Table 4

HSA/MS-U Size Distributions vs Dispersion Energy at Constant Time (10 mins)

Dispersion Energy (speed setting on vortex Genie)*	Diameter, µm (measured by SEM) Mean
2	9
4	7
6	4.4
8	3.5

Note: Data plotted in Figure 32.
*PMMA as polymer dispersant

CAB in 25 ml of ethylene dichloride in 25 x 150 mm screw cap culture tubes. The mixtures were dispersed with a Brinkman Homogenizer (PT 10-35) connected to a PT 20/TS probe generator at settings (a) 5.5 for 3.0 mins, (b) 5.5 for 10 mins, and (c) 6.5 for 10 mins. The dispersions were added dropwise to separate 500 ml round bottom flasks containing 100 ml of the 3.0% CAB polymer solution and mixed at medium speed with a magnetic stirrer. A 4.0 ml glutaraldehyde saturated toluene solution was used for cross-linking (procedure 3.11.) and the dispersion was allowed to react for 2 hrs at room temperature. The cross-linked HSA/MS-U were washed out of the polymer dispersant with acetone and dehydrated as described in procedure 3.1.1. This yielded

(1) 0.098 g, (2) 0.098 g, and (3) 0.094 g of a dry slightly agglomerated powder. Average size of the MS were determined using the SEM and were as follows: (1) 1.5 μm, (2) 1.4 μm, and (3) 0.9 μm (size distributions were tabulated and are shown in Table 5).

3.7 Hydration Effects as a Function of Cross-Link Density

3.7.1 Bovine Serum Albumin/Microspheres-Unquenched (Cross-Linked with Glutaraldehyde)

BSA samples (1) 0.150 g, (2) 0.151 g, (3) 0.150 g, and (4) 0.149 g were dissolved in 0.5 ml of water in test tubes. These solutions were dispersed in the PMMA mixture as described in procedure 3.1.1. Glutaraldehyde was used for cross-linking and was diluted with water in the following ratios: 1:1, 1:5, 1:10, and 1:20. This gave a final molar concentration of (a) 1.25 mmoles, (b) 0.5 mmoles, (c) 0.25 mmoles, and (d) 0.125 mmoles. The glutaraldehyde solutions were combined with toluene and dispersed as described in procedure 3.1.1. After addition of the glutaraldehyde satuarated toluene solutions (a) through (d) to the BSA/PMMA dispersion (1) through (4), they were mixed with a rotary mixer at r.t. for 22 hrs. After the final acetone wash and dehydration, the BSA/MS samples had a dry weight of (1) 0.125 g, (2) 0.140 g, (3) 0.135 g, and (4) 0.063 g. An aliquot of water (10.0 ml) was added to each sample and allowed to hydrate for 1.0 hr at room

Table 5

Sub-Micron: HSA/MS-U Size Distributions as a Function of Time and Energy Input in CAB Dispersant

Size/μm	% Fraction Energy 5.5 Time (5 mins)	Energy 5.5 Time (10 mins)	Energy 6.5 Time (10 mins)
0 - 0.5	0	1	6
0.5 - 0.75	0	11	49
0.75 - 1.0	7	21	13
1.0 - 1.25	19	22	19
1.25 - 1.5	30	19	10
1.5 - 1.75	23	11	3
1.75 - 2.0	13	8	0
2.0 - 4.5	8	8	0

Note: Data plotted in Figure 33

temperature. The dispersions were then centrifuged and all of the supernate was carefully removed with a pasteur pipet. BSA/MS were then re-weighed to determine water content and are as follows: (1) 0.579 g, (2) 0.881 g, (3) 1.55 g, and (4) 0.969 g; by dividing the wet weight by the dry weight the water content per mg of BSA/MS-U was determined. Average diameter of the MS for both the dry state and wet state was determined by optical microscopy; see Table 6.

3.7.2 Bovine Serum Albumin/Microspheres-Unquenched (Cross-Linked with Aldehyde-Sucrose)

BSA samples (1) 0.154 g, (2) 0.156 g, (3) 0.160 g, and (4) 0.153 g were dissolved in 0.5 ml of water in test tubes. These solutions were then dispersed in the PMMA mixture as described in procedure 3.1.1. Aldehyde-sucrose was used for cross-linking and was synthesized as described in procedure 3.2.3. Concentrations of the aldehyde-sucrose were prepared as follows: (a) 0.173 g, (b) 0.086 g, (c) 0.046 g, and (d) 0.026 g of the aldehyde were dissolved with 1.0 ml of water in test tubes. This gave final molar concentrations of (a) 0.5 mmoles, (b) 0.25 mmoles, (c) 0.125 mmoles, and (d) 0.063 mmoles. The aldehyde solutions were combined with toluene and dispersed as described in procedure 3.1.1. After addition of the aldehyde saturated toluene solutions (a) through (d) to the BSA/PMMA dispersion 1 through 4, they were mixed with a rotary mixer at r.t. for 394 hrs. After the final acetone wash and dehydration, the BSA/MS-U samples had a dry weight of (1) 0.126 g, (2) 0.126 g, (3) 0.139 g,

Table 6

Hydration of BSA/MS-U as a Function of Cross-Link Density using Glutaraldehyde

Glutaraldehyde Concentration (mmoles)	Mean Diameter Dehydrated (μm)	Mean Diameter Hydrated (μm)	mg Water Uptake/mg of BSA/HSA	% Hydration
2.50	14	17	4.6	82
0.50	13	21	6.3	86
0.25	10	23	11.5	92
0.13	17	37	15.4	94

Note: Data plotted in Figure 35

and (4) 0.085 g. After rehydration and removal of excess water as described in 3.9.1, the HSA/MS were re-weighed, yielding (1) 0.821 g, (2) 1.630 g (3) 2.309 g, and (4) 7.273 g. The water content of the BSA/MS-U samples was then determined as described in 3.9.1. Average diameters of the BSA/MS-U samples for both the dry state and wet state were determined by optical microscopy; see Table 7.

3.7.3 Bovine Serum Albumin/Microspheres-Unquenched (Cross-linked with TDC)

BSA samples (1) 0.151 g, (2) 0.157 g, (3) 0.148 g, and (4) 0.150 g were dissolved in 0.5 ml of water in test tubes. These solutions were then dispersed in the PMMA mixture as described in procedure 3.1.1. Dilutions of TDC were used for cross-linking and were prepared as follows. In four 10.0 ml volumetric flasks (a) 0.4 ml, (b) 0.2 ml, (c) 0.1 ml, and (d) 0.07 ml of TCD were diluted with toluene. This gave final molar concentrations of (a) 0.18 mmoles, (b) 0.092 mmoles, (c) 4.6 μmoles, and (d) 3.06 μmoles. One milliliter of the TDC/toluene solutions (a) through (d) was added to the albumin dispersions (1) through (4). The dispersions were then mixed with a rotary mixer at r.t. for 22 hrs. After the final acetone wash and dehydration, the BSA/MS-U samples had a dry weight of (1) 0.176 g, (2) 0.171 g, (3) 0.149 g, and (4) 0.150 g. After hydration and removal of excess water as described in 3.9.1, the BSA/MS-U samples were re-weighed; this yielded (1) 0.314 g, (2) 0.643 g, (3) 1.950 g, and (4) 3.664 g. The

Table 7

Hydration of BSA/MS-U as a Function of Cross-Link Density using Aldehyde-Sucrose

Concentration Aldehyde Sucrose (mmoles)	Mean Diameter Dehydrated (μm)	Mean Diameter Hydrated (μm)	mg Water Uptake/mg of BSA/HSA	% Hydration
0.5	19	20	6.5	87
0.25	19	27	12.9	93
0.125	18	40	16.6	94
0.063	-	-	86.1*	99

Note: Data plotted in Figure 35

*Weight of gel, no particle diameter obtainable water content of the MS was then calculated for each sample as described in procedure 3.7.1. Average diameters of the BSA/MS-U for both the dry state and wet state were determined by optical microscopy; see Table 8.

3.7.4 Bovine Serum Albumin/Microspheres-Unquenched (Cross-Linked with DCH)

BSA samples (1) 0.150 g, (2) 0.166 g, (3) 0.151 g, (4) 0.149 g, and (5) 0.160 g were dissolved in 0.5 ml of water in test tubes. These solutions were then dispersed in the PMMA mixture as described in 3.1.1. Dilutions of DCH were used for cross-linking and were prepared as follows. In five 10.0ml volumetric flasks: (a) 5.0 ml, (b) 2.0 ml, (c) 1.0 ml, (d) 0.4 ml, and (e) 0.2 ml of DCH were added and diluted with toluene. This gave final molar concentrations of (a) 5.8 mmoles, (b) 1.16 mmoles, (c) 0.58 mmoles, (d) 0.23 mmoles, and (e) 0.12 mmoles. One milliliter of the DCH/toluene solutions (a) through (e) was added to the albumin dispersions 1 through 5. The dispersions were then mixed with a rotary mixer at r.t. for 22 hrs. After the final acetone wash and dehydration, the BSA/MS samples had a dry weight of (1) 0.140 g, (2) 0.150 g, (3) 0.141 g, (4) 0.129 g, and (5) 0.144 g. After hydration and removal of excess water as described in 3.9.1, the BSA/MS-U samples were re-weighed; this yielded (1) 0.240 g, (2) 0.593 g, (3) 0.898 g, (4) 2.006 g, and (5) 2.854 g. The water content of the BSA/MS was then determined for each sample

Table 8

Hydration of BSA/MS-U as a Function of Cross-Link Density using TDC

Concentration of TDC (mmoles)	Mean Diameter Dehydrated (μm)	Mean Diameter Hydrated (μm)	mg Water Uptake/mg of BSA/HSA	% Hydration
0.18	18	19	1.8	64
0.09	18	22	3.6	78
4.60×10^{-3}	21	30	13	93
3.06×10^{-3}	16	40	24	96

Note: Data plotted in Figure 35

as described in procedure 3.7.1. Average diameter of the BSA/MS-U samples for both the dry state and wet state was determined by optical microscopy; see Table 9.

3.8 Measurements of Reactive Aldehyde Groups

3.8.1 Tritiated Leucine

HSA/MS (10 μm average diameter) were prepared as described in section 3.1.1. The cross-linked HSA/MS-U were divided into two samples. One was quenched (see Section 3.11.1) with 0.5 ml of 2-aminoethanol while the other samples was left unquenched. Tritiated leucine (New England Nuclear) specific activity 134.2 μCi/mmoles/ml, was diluted with L-leucine (Pierce). This gave a final activity of 5 μcurie/50 mmoles/ml. One milliliter of the isotope solution was added to each of three samples of 7.4 mg/ml unquenched and three samples of 8.0 mg/ml quenched MS in test tubes. The samples were incubated for 40 mins in a table top sonicator (E/MC RA Research), then washed four times with water by centrifugation (1000 RPM x 2 mins). MS pellets were resuspended in 2.0 ml of a scintillation cocktail (Aquasol New England Nuclear). From each of the solutions 1.0, 0.5, and 0.25 ml aliquotes were removed and added to scintillation counter containers. The final volumes were adjusted to 15.0 ml with additional cocktail solution. Activity was determined using a Beckman Model 230

Table 9

Hydration of BSA/MS-U as a Function of Cross-Link Density using DCH

Concentration of DCH (mmoles)	Mean Diameter Dehydrated (µm)	Mean Diameter Hydrated (µm)	mg Water Uptake/mg of BSA/HSA	% Hydration
5.8	18	20	1.7	63
1.16	21	29	3.7	79
0.58	13	28	6.4	86
0.23	17	38	16.0	94
0.12	16	50	20.0	95

Note: Data plotted in Figure 35

scintillation counter and values plotted against prepared standards, see Table 10.

3.8.2 Tritiated Leucine Binding as a Function of Particle Size

HSA/MS samples were prepared as described in procedure 3.1.1. Energy input was held constant while the vortexing time was varied (2 mins, 5 mins, and 7 mins). This gave an average particle diameter of 20 μm, 10 μm, and 3 μm. The cross-linked MS were divided into two samples. One was quenched with glycine (procedure 3.11.1); the other was left unquenched. Tritiated leucine was used for binding measurements and was prepared as described in 3.8.1. One milliliter of isotope solution was added to one sample of

Table 10

Concentration of Reactive Aldehyde Groups for HSA/MS by Binding of Tritium Labeled Leucine

Physical or Chemical Binding of Leucine	Leucine (μmoles) Bound/ml of MS	# of Leucine 10 μm MS	# Reactive CHO/MS*
HSA/MS-Q	8.80×10^{-2}	5.53×10^{7}	-
HSA/MS-U	1.29×10^{-1}	7.77×10^{7}	-
Chemically Reacted (MS-U - MS-Q)	4.01×10^{-2}	2.41×10^{7}	2.41×10^{7}

*Assume one bound leucine equals one reactive aldehyde group.

each particle size for both unquenched and quenched HSA/MS samples in test tubes and incubated for one hour in a table top sonicater. The MS samples were then washed and resuspended in a scintillation cocktail as previously described in 3.8.1. Activity was determined using a scintillation counter and values plotted against prepared standards; see Table 11.

3.8.3 Tritiated Concanavalin A (Con-A) Binding as a Function of Particle Size

HSA/MS samples were prepared as described in procedure 3.1.1. This produced MS with an average diameter of 30 μm, 12 μm, and 5 μm. The cross-linked MS were divided into two samples, quenched and unquenched. Tritiated Con-A (New England Nuclear Corp.) with a specific activity of 42.4 μCi/mmole/ml was diluted with a carrier (Con-A, Sigma) to a final activity of 1.0 μCi/0.136 μmoles/ml, in 0.01

Table 11

Leucine Binding to HSA/MS as a Function of MS Size

Mean Diameter (μm)	Leucine (μmoles) Bound/mg of HSA/MS-U	Leucine (μmoles) Bound/mg of HSA/MS-Q	Reacted Leucine μmoles/mg (U - Q)
20	6.9×10^{-3}	3.4×10^{-3}	3.5×10^{-3}
10	5.9×10^{-3}	3.7×10^{-3}	2.2×10^{-3}
3	7.1×10^{-2}	3.5×10^{-3}	3.6×10^{-3}

molar sodium phosphate buffer at pH 6.9. Two milliliter of the isotope solution was added to one sample of each particle size for both unquenched and quenched in test tubes. The samples were incubated for 1 hr in a table top sonicater, washed and resuspended in a scintillation cocktail as previously described in 3.8.1 Activity was determined using a scintillation counter and values plotted against prepared standards; see Table 12.

3.9 Chemical Analysis of Surface Properties of Microspheres

3.9.1 ESCA Determination of Residual PMMA on the Surface of Microspheres

HSA/MS unquenched were synthesized as described in procedure 3.1.1, cross-linking was with glutaraldehyde and reaction time was 22 hrs. The stabilized HSA/MS were

Table 12

Con-A Binding to HSA/MS as a Function of MS Size

Mean Diameter (μm)	Con-A (μmoles) Bound/mg of HSA/MS-U	Con-A (μmoles) Bound/mg of HSA/MS-Q	Reacted Con-A μmoles/mg (U - Q)
30	2.6×10^{-4}	2.6×10^{-4}	0
12	3.7×10^{-4}	3.2×10^{-4}	0.5×10^{-4}
5	6.7×10^{-4}	4.0×10^{-4}	2.7×10^{-4}

divided into four samples, (1) washed with acetone, (2) washed with acetone, (3) washed 4 X with acetone, and (4) washed 8 X with acetone. After the last acetone wash, each sample was then washed 4X with water, 10.0 ml volumes, to remove any excess glutaraldehyde. For controls, HSA (0.600 g) was dissolved in a test tube by addition of 2.0 ml of water. The albumin solution was cross-linked with 2.5 mmoles of glutaraldehyde. After solidification, the insoluble macroparticles were washed 4 X with acetone and 4 X with water. All of the above samples were then frozen in liquid nitrogen and lyophilized. The HSA glutaraldehyde macroparticles were ground with mortar and pestle until they were able to pass through a 200 mesh screen. The samples were mounted in an ESCA (Krato), 10 sweeps per sample and analyzed for carbon, oxygen, and nitrogen in surface atomic percent. The atomic ratios for N/C, N/O, and O/C were calculated, see Table 13.

3.10 Physical Analysis of Surface Properties of Microspheres

3.10.1 Capillary Wetting as a Function of Quenching

HSA/MS (30 μm average diameter) were prepared as described in section 3.1.1. The HSA/MS were divided into two samples. One was quenched with glycine (procedure 3.11.1); the other sample was left unquenched. HSA/MS were dehydrated with acetone and air dried. Pasteur pipets (10 cm x 1 mm I.D.) were used as the capillary column. By

Table 13

Surface Analysis for Residual PMMA
on HSA and HSA/MS with ESCA

Preparation	N/C	Atomic Ratios O/C	N/O
HSA Glutaraldehyde	0.151	0.197	0.878
HSA/MS First Wash	0.090	0.402	0.878
HSA/MS Second Wash	0.125	0.189	0.661
HSA/MS Fourth Wash	0.141	0.199	0.717
HSA/MS Eight Wash	0.146	0.199	0.826

gently pushing the pipet through a glass fiber (3 cm diameter, Gelman Type A-E) a plug was formed in the end of the column as shown in Figure 2. HSA/MS samples were loaded into the column and packed by holding the capillary tube vertically on the rubber lip of a vortex genie, then vibrating the tube for 20 to 30 seconds at a speed setting of one. Columns were packed with HSA/MS to a height of 3.0 cm from top of glass fiber plug. The capillary tube was mounted vertically and placed in a plexiglass tank (10 cm x 10 cm x 5 cm filled with water) to a depth of 1 cm. The height of the water rise up the column was

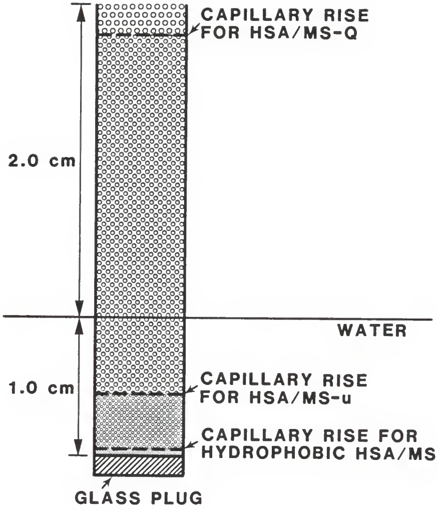

CAPILLARY RISE FOR HSA/MS-Q
2.0 cm
WATER
1.0 cm
CAPILLARY RISE FOR HSA/MS-u
CAPILLARY RISE FOR HYDROPHOBIC HSA/MS
GLASS PLUG

Figure 2. Schematic of Capillary Rise Experiment.

measured as a function of time. Both quenched and unquenched samples were run and the data taken (Table 14) were compared to HSA/MS produced by the vegetable oil method that was graciously supplied by Dr. Ken Widder of Biomolecular Systems, Inc.

3.11 Modification of Albumin/Microspheres Cross-Linked with Glutaraldehyde

3.11.1 Human Serum Albumin/Microspheres-Quenched (Q)

HSA/MS were synthesized and washed as described in procedure 3.1.1. After the last acetone wash, the HSA/MS-U pellet was resuspended with 5.0 ml of water, briefly agitated and centrifuged. This was repeated four additional times. After the last water wash, the HSA/MS pellet was resuspended in 5.0 ml of 1.0 molar glycine HCl to "quench" the residual reactive aldehyde groups. The HSA/MS glycine solution was mixed at r.t. for 22 hrs with a rotary mixer. MS were removed from the unreacted glycine by centrifugation. After decanting the glycine supernate the HSA/MS-Q pellet was resuspended in a 50 ml polypropylene centrifuge tube (Corning) with 45.0 ml of water (ph 3.0), briefly agitated and centrifuged (2000 RPM x 2 min). This procedure was repeated three times. The process was repeated again with water at pH 7.0. After the last water wash, MS were dehydrated with acetone (4 X, 100% acetone in 10.0 ml volumes) and allowed to air dry. The product was a yellowish brown powder, 115 g, 77% yield. The average size

Table 14

Hydrophilic Measurements of HSA/MS as a Function of Surface Properties by Capillary Rise

Time (mins)	HSA/MS Unquenched Height (mm)	HSA/MS Quenched Height (mm)	HSA/MS Hydrophobic Height (mm)
15	2.4	9.4	0.1
30	2.7	13.7	0.1
45	2.9	17.2	0.1
60	3.2	20.1	0.1
75	3.4	22.5	0.1
90	3.5	24.9	0.1
105	3.7	26.8	0.1
120	3.8	28.6	0.1

Note: Data plotted in Figure 42

of the MS was 26 μm in diameter as determined with optical microscopy.

3.11.2 Human Serum Albumin/Microspheres Containing Polyglutamic Acid (PGA)-Unquenched

PGA (60,000 MW, Sigma) was added into HSA/MS-U in concentrations of 11, 16, and 22 wt %. The PGA-HSA/MS were synthesized, washed and dehydrated as described in procedure 3.1.1. Samples were prepared with the following weight ratios of HSA and PGA:

	HSA	PGA
1.	0.134 g	0.017 g (0.3 μmoles)
2.	0.129 g	0.025 g (0.4 μmoles)
3.	0.199 g	0.032 g (0.5 μmoles)

The yields of the resulting cross-linked HSA-PGA/MS were as follows: (1) 0.117 g, 78%, (2) 0.134 g, 87%, and (3) 0.124 g, 82%. The average diameter of the microspheres was 34, 29, and 29 μm, respectively, as determined by optical microscopy.

3.11.3 Human Serum Albumin/Microspheres Containing Polyglutamic Acid-Quenched

HSA-PGA/MS were synthesized as described in procedure 3.11.2. The amount of added PGA was 11, 15, and 19 wt %. The PGA/MS-HSA were then quenched with glycine to "cap" residual reactive aldehyde groups. Samples were prepared with the following weight ratios of HSA and PGA:

	HSA	PGA
1.	0.133 g	0.016 g (0.30 μmoles)
2.	0.127 g	0.022 g (0.38 μmoles)
3.	0.121 g	0.029 g (0.48 μmoles)

The yields were as follows: (1) 0.115 g, 77%, (2) 0.128 g, 85%, and (3) 0.177 g, 78%. The average diameter of the microspheres were 23, 28, and 28 μm, respectively, as determined by optical microscopy.

3.11.4 Human Serum Albumin (Fatty Acid Free(FAF)) Microspheres-Unquenched

HSA(FAF) (0.145 g, Sigma) was dissolved in 0.5 ml of water in a test tube. This solution was dispersed in the PMMA solution and cross-linked with glutaraldehyde for 16 hrs as described in procedure 3.1.1. MS were washed to remove all PMMA dispersant by centrifugation with acetone (8 X) then water (4 X). After the last water wash the HSA(FAF)/MS pellet was resuspended in 10.0 ml of water. Aliquots (0.5 ml) were removed from the well shaken sample and pipeted into three pre-weighed 13 x 100 mm test tubes, then placed in a 100°C oven (National) to remove all water. Test tubes were cooled to r.t. and weighed, the average of the three weights being used to determine the weight of MS per ml of water in the 10.0 ml volume. This yield was 0.114 g, 79% of HSA-FAF/MS. The average diameter of the MS was 32 μm as determined by optical microscopy.

3.11.5 Bovine, Dog and Rabbit Serum Albumin/Microspheres-Unquenched

Bovine serum albumin (BSA) (0.150 g), dog serum albumin (DSA) (0.150 g) and rabbit serum albumin (RSA) (0.150 g) obtained from Sigma (fraction V) were dissolved in 0.5 ml of water in test tubes. The albumin/MS were then synthesized as described in procedure 3.1.1 with a cross-linking reaction time of 16 hrs. The MS were washed with acetone (8 X, 10.0 ml volumes) to remove all PMMA, then with water (4 X, 5.0 ml volumes). After the last water wash the brown pellets were resuspended in 10.0 ml of water, and weight of albumin/MS per ml was determined as described in 3.2.4. The yield was (1) 0.109 g, 73%, (2) 0.103 g, 69%, and (3) 0.128 g, 82%. Average diameter of MS was 28, 14, and 13 μm, respectively, as determined by optical microscopy.

3.11.6 Polylysine (PLY)/Microspheres-Unquenched

PLY (0.151 g, MW 11,000, 0.11 μmoles, Sigma) was dissolved in 0.5 ml of water in a test tube. This solution was dispersed in the PMMA mixture and PLY/MS were synthesized as described in 3.1.1 with a cross-linking reaction time of 2 hrs. The PLY/MS-U were washed with acetone to remove all PMMA, then with water. After the last water wash the yellow pellet was resuspended in 10.0 ml of water and weight of the PLY/MS-U were determined as described in procedure 3.2.4. This produced 0.120 g of PLY/MS-U in solution, 79% yield.

3.11.7 Fluorescent-Bovine Serum Albumin/Microspheres-Unquenched

BSA (0.130 g) and fluoresein isothicyanate (FTIC) BSA (0.020 g) were combined in a test tube and dissolved in 0.5 ml of water. The FTIC-BSA/MS were synthesized, washed and dehydrated as described in procedure 3.1.1. The product was a bright yellow powder, 0.105 g, 70% yield. The average diameter of FITC-BSA/MS was 20-30 µm, as determined by optical microscopy.

3.12 Modification of Albumin/Microspheres Cross-Linked with Tolylene 2,4-Diisocyanate

3.12.1 Bovine Serum Albumin with 14% Polyglutamic Acid-Unquenched

BSA (0.131 g) and PGA (0.022 g, 0.36 µmoles) were combined and dissolved in 0.5 ml of water in a test tube. The albumin solution was dispersed in the PMMA mixture as described in procedure 3.1.1. A 0.046 mmolar solution of TDC was prepared from 80% aqueous TDC and toluene. After mixing well, 1.0 ml of the solution was added to the albumin dispersion and mixed at r.t. for 12 hrs with a rotary mixer. BSA/MS-U were washed to remove all of the PMMA dispersant with acetone and dehydrated as described in 3.1.1. The product was a white powder, 0.146 g, 95% yield.

3.12.2 Bovine Serum Albumin with 14% Polyglutamic Acid/Microspheres-Quenched

BSA (0.132 g) and PGA (0.021 g) were combined and dissolved in 0.5 ml of water in a test tube. The albumin solution was dispersed in the PMMA mixture synthesized, washed and quenched as described in procedure 3.11.1. The product was a white powder, 0.067 g, 44% yield.

3.12.3 Bovine Serum Albumin with 12% Carboxymethyl-dextran (CMD)/Microspheres-Unquenched

CMD was synthesized by Dr. Hiroo Iwata, visiting scientist from Japan using the procedure of Pitha et al. (84). Dextran (40,000 MW, Sigma (5.0 g) was dissolved in 5.0 ml of water. This solution was added to 38 ml of 40% sodium hydroxide and 27 g of chloroacetic acid in a 125 ml erlenmeyer flask. The suspension was stirred for 12.0 hours at room temperature. After this process was repeated twice, the solution was extensively dialysed against water using membrane tubing (Spectropor) with a 6,000-8,000 MW cut off and inside tube diameter of 25.5 mm. The modified dextran was frozen in liquid nitrogen and lyophilized. Yield of product was not recorded. The carboxylic content was 4.4 μmmoles of carboxyl groups per mg of material.

BSA (0.135 g) and CMD (0.018 g) were combined and dissolved with 0.5 ml of water in a test tube. The protein/dextran solution was dispersed in the PMMA mixture and the CMD-BSA/MS were synthesized as described in procedure 3.12.1. The product was a white powder, 0.092 g, 60% yield.

3.12.4 Bovine Serum Albumin with 15% Carboxymethyldextran Microspheres-Quenched

BSA (0.139 g) and CMD (0.024 g) were combined and dissolved in 0.5 ml of water in a test tube. The protein/dextran solution was dispersed in the PMMA mixture and the CMD-BSA/MS were snythesized as described in procedure 3.12.1. The CMD-BSA/MS were washed to remove all of the PMMA dispersant, quenched and dehydrated as described in procedure 3.11.1. The product was white powder, 0.049 g, 30% yield.

3.13 Procedures for Preparing Adriamycin-Albumin/Microspheres

3.13.1 18% Adriamycin-Human Serum Albumin/Microspheres-Unquenched

Adriamycin HCL (AD) (52.3 mg) (Farmitalia Carlo ERBA) was dissolved in 25.0 ml of water, 5.0 ml of the clear dark red solution (10.46 mg AD) was combined with 9.99 mg of the HSA/MS-U (synthesized in procedure 3.1.1) in a screw cap test tube. The pH of the cloudy red mixture was adjusted from 4.00 to 5.70 by the addition of 0.1 N NaOH and mixed with a rotary mixer at 4°C in the dark for 11 hrs. The mixture was centrifuged (2000 RPM x 2 mins) and the light red supernate was carefully removed with a Pasteur pipet and saved for analysis. The dark red pellet was resuspended in 10.0 ml of water, briefly stirred, centrifuged and the supernate saved for analysis. This was repeated five times. After the last wash, the AD-HSA/MS-U were dehydrated with acetone and allowed to air dry. The product was a dark red powder 11.6 mg, 97% yield.

Concentration of A.D. in HSA/MS-U. The wash collected from the above procedure was diluted to 500 ml in a volumetric flask and analyzed with an ultraviolet visible (UV/VIS) spectrophotometer (Perkin Elemer 552) at 480 nms. This gave an adsorbance of 0.324. Using a prepared standard concentration curve (Table 15, Figure 3) this adsorbance was equal to 0.0165 mg/ml of AD in the wash. Multiplying this amount

Table 15

AD Analysis Calibration: 480 nm Adsorbance vs Concentration

Experimental Data	
Concentration (mg/ml) x 10^{-3}	Adsorbance 480 nm
4.18	0.080
8.36	0.166
10.45	0.204
20.91	0.415

Note: Data Plotted in Figure 3

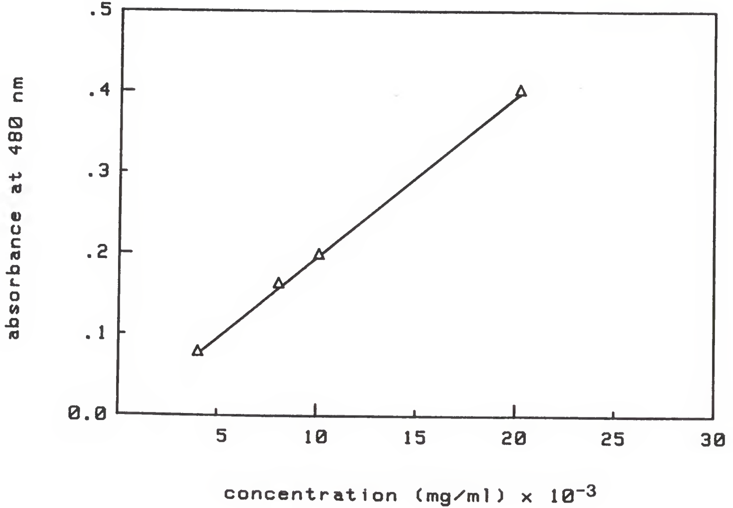


Figure 3. AD Analysis Calibration: 480 nm Absorbance vs AD Concentration.

by the dilution factor (0.0165 mg/ml x 500 ml) equals 8.27 mg of unbound AD in the supernate. The amount of AD bound to the HSA/MS-U is equal to the total AD concentration minus the unbound or 10.45 mg - 8.27 mg = 2.18 mg of bound adriamycin. The amount of bound drug was then calculated to be 18 wt %.

In Vitro Release Study: Dynamic Column Elution Method. A dynamic flow column was used to measure in vitro drug release, 2.0 ml of water was added to the dry AD-HSA/MS-U (11.61 mg), the resulting red slurry was pipeted into a 140 mm x 7 mm glass column. The ends of the column were modified with chromotography caps packed with glass wool and attached to threaded zero-volume collectors that were connected to 1.0 mm I.D. Teflon tubing. Care was taken to ensure that all the AD-HSA/MS-U were transferred into the column. The column was then placed in a circulating water bath at 37°C. Physiological saline was pumped through the column at 0.4 ml/min with an HPLC pump (ALTEX model 110 A). Fractions were collected every 30 mins for 15 hrs at 4°C. This system is shown schematically in Figure 4. Wash in each fraction was analyzed at 480 nm by UV/VIS to determine the AD concentration eluted from the AD-HSA/MS-U (Table 16). All subsequent dynamic flow in vitro release studies were performed as just described unless otherwise noted.

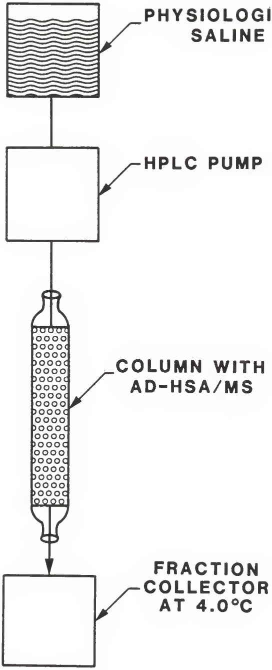


Figure 4. Schematic of In Vitro Dynamic Flow Column.

Table 16

AD Release from 18% AD-HSA/MS-U (29 μm)
Preparation 3.13.1
11.61 mg AD-HSA/MS-U (2.09 mg AD)

Drug Release	Time (hrs) 2.0	4.0	6.0	8.0	10.0	12.0	14.0
Wt AD (mg)	0.40	0.09	0.03	0.01	0.01	0.0	0.0
Cumulative Wt AD (mg)	0.40	0.49	0.52	0.53	0.54	0.54	0.54
% Released	19	24	25	25	25	25	25

Note: Data plotted in Figure 53

3.13.2 33% Adriamycin-Polyglutamic Acid (12%)-Human Serum Albumin/Microspheres-Unquenched

A volume of 5.0 ml of the stock AD solution (10.46 mg AD) prepared in procedure 3.13.1 was combined with 11.39 mg of PGA(12%)-HSA/MS-U (synthesized in procedure 3.11.2) in a screw cap test tube. The pH of the cloudy red mixture was adjusted from 4.45 to 5.84 by the addition of 0.1 N NaOH and mixed with a rotary mixer at 4°C in the dark for 11 hrs. The AD-PGA(12%)-HSA/MS-U were removed from the free drug solution and the bound concentration of AD was determined as described in procedure 3.13.1. The product was a dark red powder, 15.6 mg, 91% yield. Concentration of the bound AD to PGA(12%)-HSA/MS-U was 5.15 mg or 33 wt %.

In Vitro Release. The in vitro release of the free AD from AD-PGA(12%)/MS-U (15.6 mg) was measured as described in procedure 3.13.1; see Table 17.

3.13.3 39% Adriamycin-Polyglutamic Acid (16%)-Human Serum Albumin/Microspheres-Unquenched

A volume of 5.0 ml of the stock AD solution (10.46 mg Ad) prepared in procedure 3.13.1 was combined with 10.40 mg of PGA(16%)-HSA/MS-U (synthesized in procedure 3.11.2) in a screw cap test tube. The pH of the cloudy red mixture was adjusted from 4.32 to 5.80 by the addition of 0.1 N NaOH and mixed with a rotary mixer in the dark at 4°C for 11 hrs. AD-PGA(16%)-HSA/MS-U were washed out of the free drug solution, and concentration of the bound AD was determined as described in procedure 3.13.1. The product was a dark red powder, 11.1 mg, 65% yield. Concentration of the bound AD was 4.33 mg or 39 wt %.

In Vitro Release. The in vitro release of free AD from AD-PGA(16%)-HSA/MS-U (11.1 mg) produced in this procedure was measured as described in procedure 3.13.1; see Table 18.

3.13.4 46% Adriamycin-Polyglutamic Acid (22%)-Human Serum Albumin/Microspheres-Unquenched

A volume consisting of 5.0 ml of the stock AD solution (10.5 mg AD) prepared in procedure 3.13.1 was combined with 10.1 mg of PGA(22%)-HSA/MS-U (synthesized in procedure 3.11.2) in a screw cap test tube. The pH of the cloudy red

Table 17

AD Release from 33% AD-PGA(11%)-HSA/MS-U (34 μm)
Preparation 3.13.2
15.62 mg AD-PGA(11%)-HSA/MS-U (5.15 mg AD)

Drug Release	Time (hrs) 2.0	4.0	6.0	8.0	10.0	12.0	14.0
Wt AD (mg)	1.11	0.41	0.16	0.08	0.08	0.07	0.05
Cumulative Wt AD (mg)	1.11	1.52	1.68	1.77	1.84	1.91	1.97
% Released	22	30	33	34	36	37	38

Note: Data plotted in Figure 53

Figure 18

AD Release from 39% AD-PGA(16%)-HSA/MS-U (29 μm)
Preparation 3.13.3
11.08 mg AD-PGA(16%)-HSA/MS-U (4.33 mg AD)

Drug Release	Time (hrs) 2.0	4.0	6.0	8.0	10.0	12.0	14.0
Wt AD (mg)	0.85	0.29	0.19	0.14	0.12	0.11	0.09
Cumulative Wt AD (mg)	0.85	1.14	1.33	1.47	1.59	1.70	1.79
% Released	20	26	31	34	37	39	42

Note: Data plotted in Figure 53

mixture was adjusted from 4.43 to 5.80 by the addition of 0.1 N NaOH and mixed with a rotary mixer in the dark at 4°C for 11 hrs. AD-PGA(22%)-HSA/MS-U were washed out of the free drug solution, dehydrated and concentration of the bound AD was determined as described in procedure 3.13.1. The product was a dark red powder 14.7 mg, 78% yield. Concentration of the bound AD was 6.76 mg or 46 wt %.

In vitro release. The release of the free AD from the AD-PGA(22%)-HSA/MS-U (14.7 mg) produced in this procedure was measured as described in procedure 3.13.1; see Table 19.

3.13.5 18% Adriamycin-Human Serum Albumin/Microspheres-Quenched

AD (50.5 mg) was dissolved in 25.0 ml of water in a volumetric flask. A volume consisting of 5.0 ml of the clear dark red solution (10.1 mg AD) was combined with 10.7 mg of HSA/MS-Q (procedure 3.11.1) in a screw cap test tube. The pH of the cloudy red mixture was adjusted from 2.99 to 5.83 by the addition of 0.1 N NaOH and mixed with a rotary mixer at 4°C in the dark for 11 hrs. AD-HSA/MS-Q were removed from the free AD solution, washed, and dehydrated as described in procedure 3.13.1. The wash was saved for analysis. The product was dark red powder, 7.1 mg, 54% yield.

Concentration of AD in HSA/MS-Q. The concentration of the bound AD was determined as described in procedure 3.13.1. A standard concentration curve was prepared by

Table 19

AD Release from 46% AD-PGA(22%)-HSA/MS-U (29 μm)
Preparation 3.13.4
8.41 mg AD-PGA(22%)-HSA/MS-U (3.86 mg AD)

Drug Release	Time (hrs) 2.0	4.0	6.0	8.0	10.0	12.0	14.0
Wt AD (mg)	1.01	0.24	0.26	0.14	0.11	0.08	0.07
Cumulative Wt Ad (mg)	1.01	1.25	1.51	1.65	1.76	1.84	1.91
% Released	26	32	39	43	46	48	50

Note: Data plotted in Figure 53

making several dilutions of the AD stock solution (2.02 mg/ml) prepared for this procedure (Table 20, Figure 5). The amount of bound AD for AD-HSA/MS-Q was 1.29 mg or 18.1 wt %.

In vitro release. The release of free AD from the AD-HSA/MS-Q (7.1 mg) produced in this procedure was measured as described in procedure 3.13.1; see Table 21.

3.13.6 21% Adriamycin-Polyglutamic Acid (11%)-Human Serum Albumin/Microspheres-Quenched

A volume consisting of 5.0 ml of the stock AD solution (10.1 mg AD) prepared in procedure 3.13.5 was combined with 11.0 mg of PGA(11%)-HSA/MS-Q (synthesized in

Table 20

AD Analysis Calibration: 480 nm Adsorbance vs Concentration

Experimental Data Concentration (mg/ml) x 10^{-3}	Adsorbance 480 nm
4.04	0.080
8.07	0.164
10.09	0.200
20.18	0.403

Note: Data plotted in Figure 5

Table 21

AD Release from 18% AD-HSA/MS-Q (26 μm)
Preparation 3.13.5
7.08 mg AD-HSA/MS-Q (1.28 mg AD)

Drug Release	Time (hrs) 2.0	4.0	6.0	8.0	10.0	12.0	14.0
Wt AD (mg)	0.68	0.21	0.12	0.06	0.03	0.03	0.01
Cumulative Wt AD (mg)	0.68	0.89	1.01	1.07	1.10	1.13	1.14
% Released	53	70	77	82	86	88	89

Note: Data plotted in Figure 54

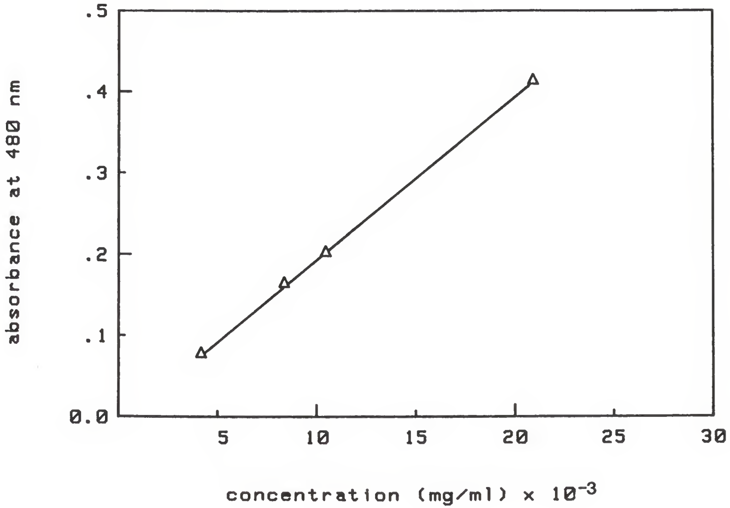


Figure 5. AD Analysis Calibration: 480 nm Absorbance vs AD Concentration.

procedure 3.11.3) in a screw cap test tube. The pH of the cloudy red mixture was adjusted from 3.46 to 5.81 by the addition of 0.1 N NaOH and mixed with a rotary mixer at 4°C in the dark for 11 hrs. AD-PGA(11%)-HSA/MS-Q were washed out of the free AD solution and dehydrated and the concentration of the bound drug was determined as described in procedure 3.13.5. The product was a dark red powder, 10.88 mg, 82% yield. Concentration of the bound AD was 2.23 mg or 21 wt %.

In vitro release. The release of the free AD from the AD-PGA(11%)-HSA/MS-Q (10.9 mg) was measured as described in procedure 3.13.1; see Table 22.

Table 22

AD Release from 21% AD-PGA(11%)-HSA/MS-Q (23 μm)
Preparation 3.13.6
10.88 mg AD-PGA(11%)-HSA/MS-Q (2.23 mg AD)

Drug Release	Time (hrs) 2.0	4.0	6.0	8.0	10.0	12.0	14.0
Wt AD (mg)	1.30	0.21	0.10	0.04	0.01	0.02	0.01
Cumulative Wt AD (mg)	1.29	1.50	1.60	1.64	1.66	1.67	1.68
% Released	58	67	72	73	74	75	75

Note: Data plotted in Figure 54

3.13.7 25% Adriamycin-Polyglutamic Acid (15%)-Human Serum Albumin/Microspheres-Quenched

A volume consisting of 5.0 ml of the stock AD solution (10.1 mg AD) prepared in procedure 3.13.5 was combined with 9.9 mg of PGA(15%)-HSA/MS-Q (synthesized in procedure 3.11.3) in a screw cap test tube. The pH of the cloudy red mixture was adjusted from 3.59 to 5.84 by the addition of 0.1 N NaOH and mixed with a rotary mixer at 4°C in the dark for 11 hrs. AD-PGA(15%)-HSA/MS-Q were washed out of the free AD solution and dehydrated and the concentration of the bound drug was determined as described in procedure 3.13.5. The product was dark red powder, 5.7 mg, 43% yield. Concentration of the bound AD was 3.33 mg or 25 wt %.

In vitro release. The release of the free AD from the AD-PGA(15%)-HSA/MS-Q (5.7 mg) was measured as described in procedure 3.13.1; see Table 23.

3.13.8 33% Adriamycin-Polyglutamic Acid (19%)-Human Serum Albumin/ Microspheres-Quenched

A volume consisting of 5.0 ml of the stock adriamycin solution (10.1 mg AD) prepared in procedure 3.13.5 was combined with 10.1 mg of PGA(19%)-HSA/MS-Q (synthesized in procedure 3.11.3) in a screw cap test tube. The pH of the cloudy red mixture was adjusted from 3.71 to 5.84 by the addition of 0.1 N NaOH and mixed with a rotary mixer at 4°C in the dark for 11 hrs. AD-PGA(19%)-HSA/MS-Q were washed out of the free AD solution and dehydrated and the concentration of the bound drug was determined as

Table 23

AD Release from 25% AD-PGA(15%)-HSA/MS-Q (28 μm)
Preparation 3.13.7
5.70 mg AD-PGA(15%)-HSA/MS-Q (1.43 mg AD)

Drug Release	Time (hrs) 2.0	4.0	6.0	8.0	10.0	12.0	14.0
Wt AD (mg)	0.77	0.08	0.02	0.02	0.02	0.02	0.01
Cumulative Wt AD (mg)	0.77	0.85	0.87	0.89	0.91	0.93	0.94
% Released	54	59	61	62	64	65	66

Note: Data plotted in Figure 54

described in procedure 3.13.5. The product was a dark red powder, 7.9 mg ,53% yield. Concentration of the bound drug was 4.9 mg or 33 wt %.

In vitro release. The release of the free AD from the AD-PGA(19%)-HSA/MS-Q (7.9 mg) was measured as described in 3.13.1; see Table 24.

3.13.9 Ion Exchange Release Properties of Adriamycin-Albumin/Microspheres-Quenched

AD was bound to (1) HSA/MS-Q (quenched with 2-aminoethanol), (2) HSA/MS-Q (quenched with glycine), and (3) PGA(15%)-HSA/MS-Q. MS were washed out of the free drug solution and dehydrated and the concentration of the bound AD was determined as described in procedure 3.14.1.

Table 24

AD Release from 33% AD-PGA(19%)-HSA/MS-Q (24 μm)
Preparation 3.13.8
5.70 mg AD-PGA(15%)-HSA/MS-Q (2.58 mg AD)

Drug Release	Time (hrs) 2.0	4.0	6.0	8.0	10.0	12.0	14.0
Wt AD (mg)	0.77	0.20	0.14	0.09	0.08	0.07	0.07
Cumulative Wt AD (mg)	0.77	0.97	1.11	1.19	1.27	1.34	1.40
% Released	30	38	43	46	49	52	54

Note: Data plotted in Figure 54

After dehydration, the AD-MS had a yield of (1) 8.28 mg, 75%, (2) 7.98 mg, 77%, and (3) 9.50 mg, 57%. Concentration of the bound drug for each sample was (1) 1.4 mg or 14%, (2) 1.6 mg or 20%, and (3) 4.8 mg or 28%.

In vitro release. The AD-albumin/MS samples were separately loaded in the release column as described in procedure 3.13.1. Water was used as the mobile phase for the first 5.0 hrs, then exchanged for physiological saline for the remainder of the experiment (for release data see Tables 25, 26, and 27).

Table 25

AD Release from 14% AD-HSA/MS-Q (Aminoethanol) (25 μm)
Preparation 3.13.9
8.28 mg AD-HSA/MS-Q (1.16 mg AD)

Drug Release			Time (hrs)				
	2.0	4.0	6.0	8.0	10.0	12.0	14.0
Wt AD (mg)	0.16	0.12	0.17	0.30	0.05	0.02	0.0
Cumulative Wt AD (mg)	0.16	0.28	1.45	0.75	0.80	0.83	0.83
% Released	14	24	39	65	70	71	71

Note: Data plotted in Figure 57

Table 26

AD Release from 20% AD-HSA/MS-Q (Glycine) (25 μm)
Preparation 3.13.9
7.98 mg AD-HSA/MS-Q (1.59 mg AD)

Drug Release			Time (hrs)				
	2.0	4.0	6.0	8.0	10.0	12.0	14.0
Wt AD (mg)	0.14	0.12	0.38	0.18	0.08	0.08	0.08
Cumulative Wt AD (mg)	0.14	0.26	0.64	0.82	0.90	0.98	1.06
% Released	8.5	16.1	40	52	56	60	67

Note: Data plotted in Figure 57

Table 27

AD Release from AD-PGA(15%)-HSA/MS-Q (28 μm)
Preparation 3.13.9
9.54 mg AD-PGA(15%)-HSA/MS-Q (2.67 mg AD)

Drug Release	Time (hrs) 2.0	4.0	6.0	8.0	10.0	12.0	14.0
Wt AD (mg)	0.08	0.03	0.23	0.79	0.25	0.13	0.12
Cumulative Wt AD (mg)	0.08	0.11	0.34	1.13	1.38	1.51	1.63
% Released	3	4	13	43	52	58	61

Note: Data plotted in Figure 57

Table 28

AD Analysis Calibration: 480 nm Adsorbance
vs AD Concentration

Experimental Data	
Concentration (mg/ml) x 10^{-3}	Adsorbance 480 nm
4.29	0.060
5.37	0.108
10.74	0.220
21.47	0.436

Note: Data plotted in Figure 6

3.13.10 Binding of Adriamycin as a Function of pH with Polyglutamic Acid (12%)-Human Serum Albumin/Microspheres-Quenched

HSA/MS-Q were produced containing 12% of PGA (70,000 MW) as described in procedure 3.11.3. AD (26.9 mg) was dissolved with 25.0 ml of water; 5.0 ml aliquots (5.9 mg AD) of the solution were added to screw cap test tubes (1) through (5) containing (1) 6.4 mg, (2) 6.2 mg, (3) 5.5 mg, (4) 5.5 mg, and (5) 5.9 mg of PGA(12%)-HSA/MS-Q. The pH of the cloudy red solutions were adjusted in the 5 samples from (1) 4.58 to 3.11, (2) 4.46 to 4.06, (3) 4.48 to 5.05, (4) 4.64 to 6.00, and (5) 4.53 to 7.00 by the addition of 0.1 N HCl for samples (1) through (2) and 0.1 N NaOH for samples (3) through (5). The solutions were mixed with a rotary mixer at 4°C in the dark for 19 hrs.

Concentration of bound AD as a function of pH. The concentration of the bound AD for each sample was determined as described in procedure 3.13.1. A standard concentration curve was prepared by making several dilutions of the AD stock solution (1.08 mg/ml) prepared for this procedure. These values are shown in Table 28, Figure 6. Concentration of the bound AD vs pH was tabulated; see Table 29.

3.13.11 In Vitro Release of Adriamycin from Albumin/Microspheres in a Static System

PGA(10%)HSA/MS-Q containing AD were synthesized as described in procedure 3.10.10. MS were washed out of the free AD solution and dehydrated and the concentration of the bound AD was determined as described in procedure 3.13.1.

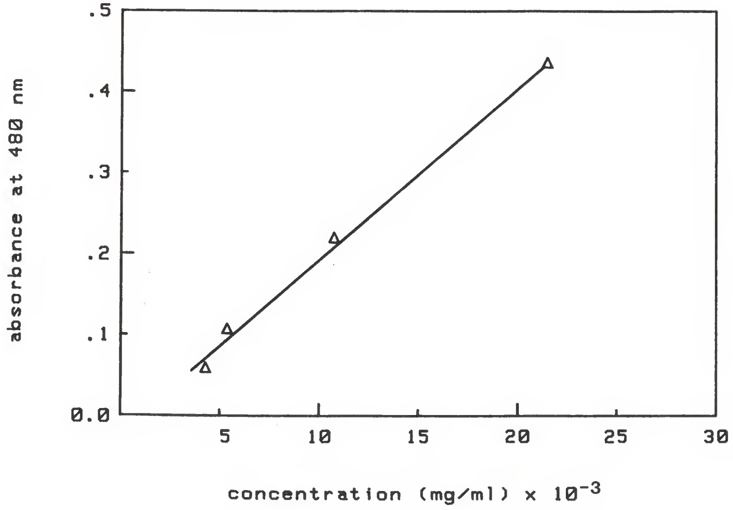


Figure 6. AD Analysis Calibration: 480 nm Absorbance vs AD Concentration.

Table 29

Amount of Bound AD to PGA(12%-HSA)/MS-Q
as a Function of pH

pH	Weight % Bound AD
3.1	14
4.1	17
5.1	24
6.0	32
7.0	31

Note: Data plotted in Figure 52

The product was a dark red powder, 6.1 mg, 75% yield. The concentration of the bound drug was 2.6 mg or 31.7 wt %.

In vitro release. The AD-PGA(10%)-HSA/MS were combined with 2.0 ml of physiological saline in a screw cap test tube and placed in a 37°C shaker water bath. Every 30 mins, the cloudy red mixture was centrifuged and 50 μl was removed from the clear red supernate, diluted in 5.0 ml of physiological saline. The concentration of the released AD was then determined as previously described in procedure 3.13.1. This procedure was repeated until the amount of release drug remained constant (i.e. no more AD released). The AD-PGA(10%)-HSA/MS-Q were then washed (5 X with 10.0 ml volumes of water) to remove all free AD. After the last water wash was decanted, the MS were resuspended with 2.0 ml of saline and the process duplicated for the remainder of the experiment (15 hrs).

3.14 In Vivo Studies

3.14.1 Toxicity of Adriamycin-Polyglutamic Acid (15%)-Human Serum Albumin/Microspheres-Quenched in CD-1 Mice

AD-PGA(15%)-HSA/MS-Q were synthesized according to the procedure described in 3.13.7. The product was a dark red powder with 27 wt % bound AD and total yield of 76%. MS had a size range of 20 to 40 μm.

CD-1 white female mice, 5-7 weeks of age, weighing 30-33 g were injected by the interpertinal (i.p.) route with AD at concentrations of 0.6 mg and 1.5 mg dissolved in 0.5 ml of sterile saline. A second group of animals were injected with AD-PGA(15%)-HSA/MS-Q with 0.6, 1.5, and 2.5 mg of bound AD. For each concentration of the free and bound drug, 5 mice were used. PGA(15%)-HSA/MS-Q without AD was used for a control group and injected in equivalent weight amounts. Animals were then observed over a two month period; fatalities were recorded for each group and tabulated; see Table 30.

3.14.2 In Vitro-Degradation and Immobilization Studies of Adriamycin-Albumin/Microspheres and Fluorescent-Albumin/Microspheres

AD-PGA(15%)-HSA/MS-Q (50 mg) of AD were suspended in 1.0 ml of sterile water in a 1.0 cc syringe. CD-1 white

Table 30

Toxicity of AD-PGA-HSA/MS-U in CD-1 Mice
by i.p. Injection

Preparation and Dose	Survival from Time of Administration				
	7 Days	14 Days	21 Days	28 Days	35 Days
AD (0.6 mg)	5/5	1/5	1/5	1/5	1/5
AD (1.5 mg)	5/5	0/5	-	-	-
AD-PGA-HSA-MS (AD 0.6 mg) (2.22 mg MS)	5/5	5/5	5/5	5/5	5/5
AD-PGA-HSA-MS (AD 1.5 mg) (4.44 mg MS)	5/5	5/5	5/5	5/5	5/2
AD-PGA-HSA-MS (AD 2.5 mg) (9.26 mg MS)	5/5	4/5	1/5	0/5	-
PGA-HSA/MS (2.22 mg MS)	5/5	5/5	5/5	5/5	5/5
PGA-HSA/MS (4.44 mg MS)	5/5	5/5	5/5	5/5	5/5
PGA-HSA/MS (9.26 mg MS)	5/5	5/5	5/5	5/5	5/5

Note: Date plotted in Figure 59

female mice, 5-7 weeks of age, weighing 30-33 g were injected in the right hind leg muscle with a 0.2 ml dose of the AD-PGA(15%)-HSA/MS-Q. At times of 1 day, 3 days, 1 week, 2, 3, and 4 weeks, the animals were sacrificed, muscle removed and fixed in 8% phosphate buffered glutaraldehyde. Histological slides were made and examined with an optical microscope under fluorescent light. This procedure was repeated with FTIC-BSA/MS-U synthesized in procedure 3.11.7.

3.15 Procedures for Preparing Bleomycin-Albumin/Microspheres

Bleomycin Sulfate (BLM), obtained as a gift from Bristol Laboratories, was supplied in 10.0 ml sealed glass vials containing between 8 and 9 mg of a lyophilized white powder that was amorphous in texture. To measure the exact concentration of the BLM, the glass vials were opened in the exhaust hood and the drug dissolved by the addition of known volumes of water. The concentration was then determined with the UV/VIS at an adsorbance of 294 nm and using an extinction coefficient of 12.15 ml/mg (85).

3.15.1 30% Bleomycin-Human Serum Albumin/Microspheres-Unquenched

A stock solution of BLM was prepared containing 66.7 mg BLM in 25.0 ml of water. An aliquot consisting of 5.0 ml of the clear solution (13.3 mg BLM) was combined with 11.3 mg HSA/MS-U (synthesized as described in procedure 3.1.1) in a screw cap test tube. The pH of the brown cloudy mixture was adjusted from 4.23 to 5.97 by the addition of 0.1 N NaOH and mixed with a rotary mixer in the dark at 4°C for 7 hrs. BLM-HSA/MS-U were washed free of the unbound BLM and supernate saved for analysis as described in procedure 3.13.1. After the last wash, the MS were dehydrated with acetone and allowed to air dry. The product was a brown powder, 11.7 mg, 73% yield.

Concentration of BLM in HSA/MS-U. The wash collected from the above procedure was diluted to 500 ml in a volumetric flask and analyzed by UV/VIS at 294 nm. This gave an absorbance of 0.208. Using the equation

$$A = B \times C \times \Sigma$$

where A is the absorbance in nm, Σ is the extinction coefficient, B the path length of the cell and C the concentration of the solution. Typical calculations for the amount of unbound drug from A would be

$$C = \frac{A}{B} = \frac{0.208}{(12.15\ \text{ml/mg})\ \text{cm}} = 0.017\ \text{mg/ml} \times 500\ \text{ml}$$

$$= 8.56\ \text{mg BLM}$$

The amount of bound drug was then equal to total BLM minus unbound or 13.34 mg - 8.56 mg = 4.78 mg of bound BLM. The amount of bound drug was then calculated to be 30 wt %.

In vitro release. The release of free BLM from BLM-HSA/MS-U (11.7 mg) was performed using the in vitro dynamic flow column described in procedure 3.13.1. Concentration of released drug was determined as just described; for results, see Table 31.

3.15.2 31% Bleomycin-Polyglutamic Acid (9%)-Human Serum Albumin/Microspheres-Unquenched

An aliquot consisting of 5.0 ml of the BLM stock solution (13.34 mg BLM) prepared in procedure 3.15.1 was combined with 12.7 mg of PGA(9%)-HSA/MS-U (synthesized as described in procedure 3.11.2) in a screw cap test tube. The pH of the mixture was adjusted from 4.71 to 6.00 by the addition 0.1 N NaOH and mixed at 4°C in the dark for 7 hrs. The BLM-PGA(9%)-HSA/MS-U were removed from solution and concentration of the bound drug was determined as described in procedure 3.15.1 The product was a brown powder, 13.2 mg, 72% yield. Concentration of the bound drug was 5.69 mg or 31 wt %.

In vitro release. The in vitro release of free BLM from BLM-PGA(9%)-HSA/MS-U (13.15 mg) was measured as described in procedure 3.15.1; for results see Table 32.

3.15.3 30% Bleomycin-Polyglutamic Acid (14%)-Human Serum Albumin/Microspheres-Unquenched

An aliquot consisting of 5.0 ml of the stock BLM solution (13.3 mg BLM) prepared in procedure 3.15.1 was combined with 12.8 mg of PGA(14%)-HSA/MS-U (synthesized as

Table 31

BLM Release from 29% BLM-HSA/MS-U (29 μm)
Preparation 3.15.1
11.67 mg BLM-HSA/MS-U (3.50 mg BLM)

Drug Release	Time (hrs) 2.0	4.0	6.0	8.0	10.0	12.0	14.0
Wt BLM (mg)	1.29	0.06	0.04	0.03	0.02	0.02	0.01
Cumulative Wt BLM (mg)	1.29	1.35	1.39	1.42	1.44	1.46	1.47
% Released	37	39	40	41	41	42	42

Note: Data plotted in Figure 65

Table 32

BLM Release from 31% BLM-PGA(9%)-HSA/MS-U (27 μm)
Preparation 3.15.2
13.15 mg BLM-PGA(9%)-HSA/MS-U (4.08 mg BLM)

Drug Release	Time (hrs) 2.0	4.0	6.0	8.0	10.0	12.0	14.0
Wt BLM (mg)	2.32	0.09	0.06	0.03	0.04	0.02	0.0
Cumulative Wt BLM (mg)	2.32	2.41	2.47	2.50	2.54	2.56	2.56
% Released	57	59	61	61	62	63	63

Note: Data plotted in Figure 65

described in procedure 3.11.2) in a screw cap test tube. The pH of the cloudy mixture was adjusted from 4.83 to 6.00 by the addition of 0.1 N NaOH and mixed with a rotary mixer at 4°C in the dark for 7 hrs. BLM-PGA(14%)-HSA/MS-U were washed out of the free drug solution and dehydrated and the concentration of the bound drug was determined as described in procedure 3.15.1. The product was a brown powder, 13.8 mg, 75% yield. Concentration of the bound BLM was 5.56 mg or 30 wt %.

In vitro release. The in vitro release of the free BLM from BLM-PGA(14%)-HSA/MS-U (13.8 mg) was measured as described in procedure 3.15.1; for results see Table 33.

3.15.4 23% Bleomycin-Human Serum Albumin/Microspheres-Quenched

A stock solution of BLM was prepared containing 62.7 mg dissolved with 25.0 ml of water in a volumetric flask. An aliquot consisting of 5.0 ml (12.6 mg) was combined with 11.9 mg of HSA/MS-Q (synthesized as described in procedure 3.11.1) in a screw cap test tube. The pH of the mixture was adjusted from 3.95 to 5.95 by the addition 0.1 NaOH and mixed with a rotary mixer at 4°C in the dark for 19 hrs. The BLM-HSA/MS-Q were removed from solution and dehydrated and the concentration of bound drug was determined as described in procedure 3.15.1. The product was a brown powder, 11.8 mg, 76% yield. Concentration of the bound drug was 3.58 mg or 23 wt %.

Table 33

BLM Release from 30% BLM-PGA(14%)-HSA/MS-U (31 μm)
Preparation 3.15.3
13.79 mg BLM-PGA(14%)-HSA/MS-U (4.14 mg BLM)

Drug Release	Time (hrs) 2.0	4.0	6.0	8.0	10.0	12.0	14.0
Wt BLM (mg)	2.41	0.06	0.03	0.02	0.02	0.01	0.01
Cumulative Wt BLM (mg)	2.41	2.47	2.50	2.52	2.54	2.55	2.56
% Released	58	60	60	61	61	62	62

Note: Data plotted in Figure 66

In vitro release. The in vitro release of the free BLM from BLM-HSA-/MS-Q (11.8 mg) was measured as described in procedure 3.15.1; for results see Table 34.

3.15.5 28% Bleomycin-Polyglutamic Acid (9%)-Human Serum Albumin/Microspheres-Quenched

An aliquot consisting of 5.0 ml of the stock BLM solution (12.6 mg BLM) prepared in procedure 3.15.4 was combined with 13.9 mg of PGA(9%)-HSA/MS-Q (synthesized as described in procedure 3.11.3) in a screw cap test tube. The pH of the mixture was adjusted from 3.68 to 5.98 by the addition of 0.1 N NaOH and mixed with a rotary mixer at 4°C in the dark for 19 hrs. The BLM-PGA(9%)-HSA/MS were washed out of the free BLM solution and dehydrated and the

Table 34

BLM Release from 23% BLM-HSA/MS-Q (29 μm)
Preparation 3.15.4
11.81 mg BLM-HSA/MS-Q (2.71 mg)

Drug Release	Time (hrs) 2.0	4.0	6.0	8.0	10.0	12.0	14.0
Wt BLM (mg)	1.16	0.04	0.02	0.02	0.01	0.0	0.0
Cumulative Wt BLM (mg)	1.16	1.20	1.22	1.24	1.25	1.25	1.25
% Released	43	44	45	46	46	46	46

Note: Data plotted in Figure 67

concentration of the bound drug was determined as described in procedure 3.15.1. The product was a brown powder, 16.2 mg, 84% yield. Concentration of the bound drug was 5.51 mg or 28 wt %.

In vitro release. The release of the free BLM from BLM-PGA(9%)-HSA/MS-Q (16.2 mg) was measured as described in procedure 3.15.1; for results; see Table 35.

3.15.6 29% Bleomycin-Polyglutamic Acid (14%)-Human Serum Albumin/Microspheres-Quenched

An aliquot consisting of 5.0 ml of the stock BLM solution (12.6 mg BLM) prepared in procedure 3.15.4 was combined with 12.1 mg of PGA(14%)-HSA/MS-Q (synthesized as described in procedure 3.11.3) in a screw cap test tube.

Table 35

BLM Release from 28% BLM-PGA(9%)-HSA/MS-Q (27 μm)
Preparation 3.15.5
16.20 mg BLM-PGA(9%)-HSA/MS-U (4.54 mg BLM)

Drug Release	Time (hrs) 2.0	4.0	6.0	8.0	10.0	12.0	14.0
Wt BLM (mg)	2.53	0.05	0.02	0.01	0.01	0.0	0.0
Cumulative Wt BLM (mg)	2.53	2.58	2.60	2.61	2.62	2.62	2.62
% Released	56	57	57	57	58	58	58

Note: Data plotted in Figure 67

The pH of the mixture was adjusted from 3.65 to 5.97 by the addition of 0.1 N NaOH and mixed with a rotary mixer at 4°C in the dark for 19 hrs. The BLM-PGA(14%)-HSA/MS-Q were washed out of the free BLM solution and dehydrated and the concentration of the bound drug was determined as described in procedure 3.15.1. The product was a brown powder, 13.2 mg, 78% yield. Concentration of the bound drug was 4.98 mg or 29 wt %.

In vitro release. The release of the free BLM from BLM-PGA(14%)-HSA/MS-Q (13.2 mg) was measure as described in procedure 3.15.1; for release results; see Table 36.

Table 36

BLM Release from 29% BLM-PGA(14%)-HSA/MS-Q (31 μm)
Preparation 3.15.6
13.21 mg BLM-PGA(14%)-HSA/MS-U (3.83 mg BLM)

Drug Release	Time (hrs) 2.0	4.0	6.0	8.0	10.0	12.0	14.0
Wt BLM (mg)	2.15	0.04	0.02	0.01	0.0	0.0	0.0
Cumulative Wt BLM (mg)	2.15	2.19	2.21	2.22	2.22	2.22	2.22
% Released	56	57	58	59	59	59	59

Note: Data plotted in Figure 68

3.16 Procedures for Preparing Gentamycin-Albumin/Microspheres

Gentomycin sulfate (GMC) has as aminoglycoside structure with no detectable absorbance in either the ultraviolet or visable range. In order to quantitate the concentration of GMC for the drug binding experiments, a modification of the Barends et al. (86) procedure was used. This involves the modification of GMC with 1-fluoro-2,4-dinitrobenzene (FDNB) which produces a chromophor. The reaction products are chromatographed on a microparticulate C18 reverse-phase column at 365 nm.

Briefly, the procedure is as follows, 0.5 ml of the GMC sample is combined with 0.075 ml of 1.0 N NaOH and 2.0 ml of acetonitrile in a test tube. After vortexing, 1.5 ml of the solution is removed and combined with 0.15 ml of FDNB in a 2.0 ml glass ampoule. The ampoule is flame sealed and incubated at 80°C for 45 mins. Then 0.035 ml was removed with a microsyringe and injected into the chromatographic system (Perkin Elmer Series 3B). The system consisted of solvent delivery unit with 0.020 ml injection loop with variable UV/VIS absorbance detector set at 365 nm. Column material was C18 (30 cm x 3.9 mm I.D., particle size 10 μm). Retention times and peak areas were measured with a chromatographic data system (Sigma 10). The mobile phase consisted of 1 g/l tris-(hydroxymethyl)aminomethane in water, (300 ml/l) adjusted with 1.0 N HCl to pH 7.0, and acetonitrile (700 ml/l). This solution was passed through a 0.2 μm filter and de-aerated ultrasionically. The flow-rate was maintained at 1.0 ml/minute. The GMC chromatograph shown in Figure 7 are for typical GMC. Standard concentration curves were assayed to quantitate amounts of GMC in the samples and calculated by determining the area under the curves (peaks labeled 7.24 and 8.26 mins) for the GMC chromatograph.

3.16.1 12% Gentamycin-Human Serum Albumin/Microspheres-Unquenched

A solution of GMC was prepared containing 257.9 mg dissolved with 25.0 ml of water in a volumetric flask. An aliquot consisting of 5.0 ml of the clear solution (51.6 mg) was combined with 50.5 mg of the dehydrated HSA/MS-U (synthesized as described in procedure 3.1.1) in a screw cap

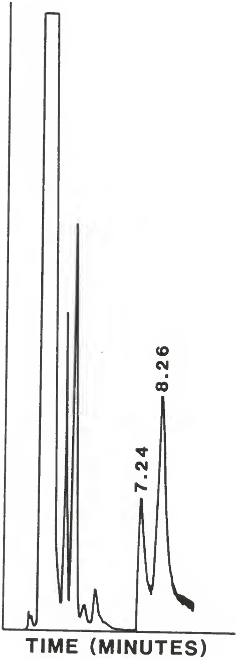


Figure 7. HPLC Chromatograph for GMC.

test tube. The pH of the brown cloudy mixture was adjusted from 5.18 to 5.70 by the addition of 0.1 N NaOH and mixed at 4.0 C in the dark for 12 hrs. GMC-HSA/MS-U were washed free of the unbound GMC and the supernate saved for analysis as described in procedure 3.13.1. After the last wash, the MS were dehydrated with acetone and allowed to air dry. The product was a brown powder, 52.4 mg, 92% yield.

Concentration of bound GMC. The wash collected in the above procedure was diluted to 250 ml in a volumetric flask and the concentration of GMC in the wash was analyzed by the Bavends method as described. This gave a combined peak area of 1.44 cm. Using the prepared standard concentration curve (Table 37, Figure 8), this area was equal to 0.036 mg/ml x 1250 ml) = 45.0 mg of unbound GMC in the supernate. The amount of GMC bound to HSA/MS-U is equal to the total GMC concentration minus the unbound or 51.60 mg - 45.0 mg = 6.60 mg of bound GMC. The amount of bound drug was then calculated to be 12 wt %. Release study were not performed on this sample.

3.16.2 16% Gentamycin-Polyglutamic Acid (17%)-Human Serum Albumin/Microspheres-Unquenched

A solution of GMC was prepared containing 252.02 mg dissolved in 25.0 ml of water in a volumetric flask. An aliquot consisting of 10.0 ml of the clear solution (100.8 mg) was combined with 119.06 of PGA(17%)-HSA/MS-U (synthesized as described in procedure 3.11.3) in a screw cap test tube. The pH of the brown cloudy mixture was

Table 37

Calibration for HPLC Analysis of GMC:
HPLC Area vs Concentration

Experimental Data	
Concentration (mg/ml) x 10^{-3}	Area (cm^2)
20.6	0.672
41.4	1.680
103.1	5.801

Note: Data plotted in Figure 8

adjusted from 5.30 to 5.74 by the addition of 0.1 N NaOH and mixed with a rotary mixer at 4°C in the dark for 12 hrs. GMC-PGA(17%)-HSA/MS-U were removed from the free drug solution by centrifugation, washed and dehydrated as described in 3.16.1. The supernate was saved for analysis. The product was a brown powder, 113.6 mg, 81% yield.

Concentration of GMC in PGA (17%)-HSA/MS-U. The concentration of the bound GMC was determined as described in procedure 3.16.1. A standard concentration curve was prepared by making several dilutions of the GMC solution (10.08 mg/ml) used in this procedure (Table 38, Figure 9). The amount of bound drug was calculated to be 20 mg of GMC or 16 wt %. In vitro release was not measured in this sample.

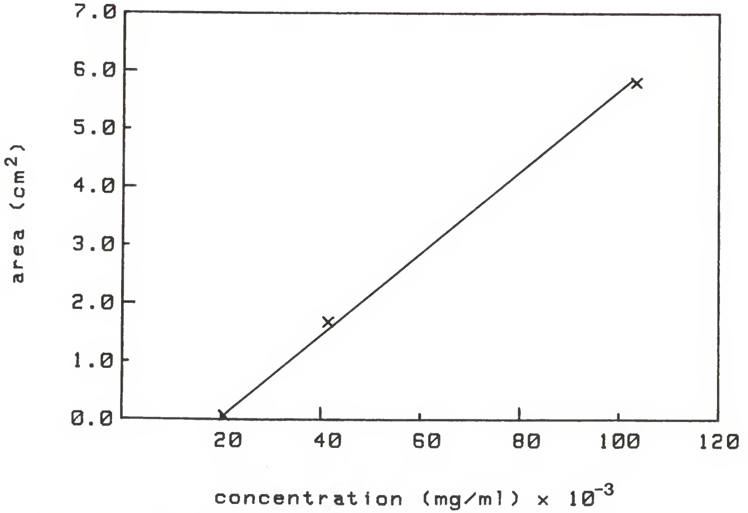


Figure 8. Calibration for HPLC Analysis of GMC: HPLC Area vs Concentration.

Table 38

Calibration for HPLC Analysis of GMC:
HPLC Area vs Concentration

Experimental Data Concentration (mg/ml) x 10^{-3}	Area (cm^2)
20.2	0.618
40.3	1.600
100.8	3.725

Note: Data plotted in Figure 9

3.16.3 18% Gentamycin-Polyglutamic Acid (19%)-Bovine Serum Albumin/Microspheres-Unquenched

A solution of GMC was prepared containing 252.9 mg dissolved in 25.0 ml of water in a volumetric flask. An aliquot consisting of 10.0 ml of the clear solution (101.2 mg GMC) was combined with 127.7 mg of the dehydrated PGA(19%)-BSA/MS-U (synthesized by a modification of the procedure described in 3.11.5) in a screw cap test tube. The pH of the brown cloudy mixture was adjusted from 5.12 to 5.83 by the addition of 0.1 N NaOH and mixed with a rotary mixer at 4°C in the dark for 12 hrs. GMC-PGA(19%)-BSA/MS-U were removed from the free drug solution by centrifugation washed and dehydrated as described in 3.16.1. The supernate was saved for analysis, the final yield was not calculated.

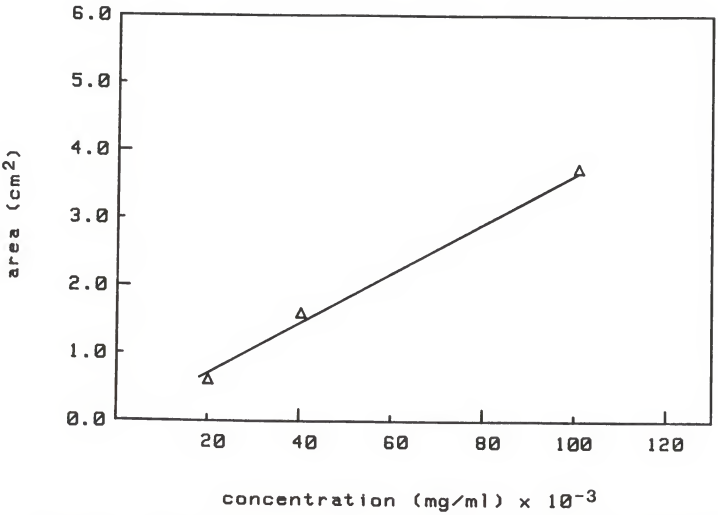


Figure 9. Calibration for HPLC Analysis of GMC: HPLC Area vs Concentration.

Concentration of GMC in PGA (19%)-BSA/MS-U. The concentration of the bound GMC was determined as described in procedure 3.16.1. A standard concentration curve was prepared by making several dilutions of the GMC solution (10.1 mg/ml) used in the procedure (Table 39, Figure 10). The amount of bound drug was calculated to be 22.38 mg or 18 wt %.

In vitro release. The in vitro release of GMC from GMC-PGA(19%)-BSA/MS-U was performed as described in procedure 3.14.1. Concentration of the free drug in the collected fractions was analyzed as described in 3.17.1. For release data; see Table 40.

Table 39

Calibration for HPLC Analysis of GMC:
HPLC Area vs Concentration

Experimental Data	
Concentration (mg/ml) x 10^{-3}	Area (cm^2)
20.2	0.429
41.8	1.134
102.1	3.325

Note: Data plotted in Figure 10

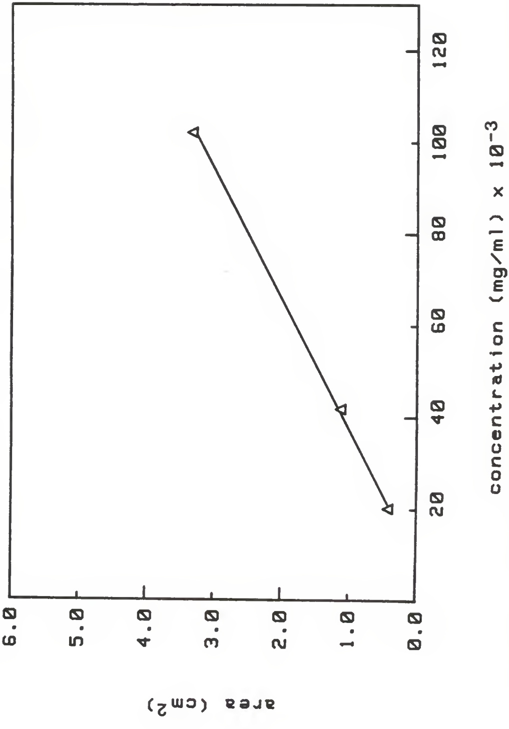


Figure 10. Calibration for HPLC Analysis of GMC: HPLC Area vs Concentration.

Table 40

GMC Release from (18%) GMC-PGA(19%)-BSA/MS-U (18 μm)
Preparation 3.17.3
16.37 mg GMC-PGA(19%)-BSA/MS-U)

Drug Release	Time (hrs) 2.0	4.0	6.0	8.0	10.0	12.0	14.0
Wt GMC (mg)	1.66	0.49	0.16	0.01	0.0	0.0	0.0
Cumulative Wt GMC (mg)	1.66	2.15	2.31	2.32	2.32	2.32	2.32
% Released	58	75	80	81	81	81	81

Note: Data plotted in Figure 70

3.17 Procedures for Preparing Streptomycin-Albumin/Microspheres

Streptomycin sulfate (STM), obtained from Sigma, has no detectable absorbance in either the ultraviolet or visible range. As with GMC, STM has to be modified in order to quantitate the amounts of STM bound to the MS. The mannitol procedure by Grove and Randall (87) was used.

Briefly, the procedure is as follows, to a 25 ml volumetric flask transfer 1.0 ml of a 5.0 mg/ml solution of STM. To this, add 2.0 ml of 1.0 N NaOH and heat in boiling water for 10 minutes. Cool in ice water for 3 mins then add 2.0 ml of 1.2 N HCl and 5.0 ml of 0.25% ferric chloride. Make to volume with water. Read absorbance of colored solution at 550 nm with a UV/VIS spectrophotometer. Unknown concentrations were plotted against a prepared standard concentration curves.

3.17.1 18% Streptomycin-Polyglutamic Acid (14%)-Bovine Serum Albumin/Microspheres-Unquenched

STM (259.6 mg) was dissolved with 25.0 ml of water. Five milliliters of this clear solution (51.9 mg STM) was combined with 48.3 mg of PGA(14%)-BSA/MS-U (synthesized as described in procedure 3.12.1) in a screw cap test tube. The pH of the cloudy white mixture was adjusted from 4.23 to 5.79 by the addition of 0.1 N NaOH and mixed with a rotary mixer at 4.0 C in the dark for 18 hrs. the mixture was centrifuged (2000 RPM x 2 mins) and the clear supernate was removed and saved for analysis. The STM-PGA(14%)-BSA/MS-U were removed from the free drug solution, washed and dehydrated. The product was a white powder, 27.5 mg, 47% yield.

Concentration of STM in PGA (14%)-BSA/MS-U. From the collected wash, 1.0 ml was transferred to a 25 ml volumetric flask and analyzed by the mannitol method as previously described. This gave an absorbance of 0.427 at 550 nm. Using a prepared standard concentration curve (Table 41, Figure 11). This absorbance was equal to 0.33 mg/ml of free STM in the supernate. Multiplying this amount by the dilution factor (0.33/mg x 125 ml) = 41.3 mg of unbound STM in the supernate. The amount of bound STM was equal to the

Table 41

STM Analysis Calibration: 550 nm
Absorbance vs Concentration

Experimental Data	
Concentration (mg/ml) x 10^{-3}	Absorbance 55- m,
41.53	0.055
83.06	0.103
124.12	0.160
248.24	0.315
372.36	0.472

Note: Data plotted in Figure 11

total STM concentration minus the unbound or 51.9 mg - 41.3 mg = 10.6 mg or 18 wt % of bound STM. In vitro release studies were performed using the dynamic flow column, but no STM was detectable, see discussion.

3.17.2 25% Streptomycin-Polyglutamic Acid (14%)-Bovine Serum Albumin/Microspheres-Quenched

A volume of 5.0 ml of the stock STM solution (51.9 mg STM) prepared in procedure 3.18.1, was combined with 38.8 mg of the PGA(14%)-BSA-PGA/MS-Q (synthesized as described in 3.12.1) in a screw cap test tube. The pH of

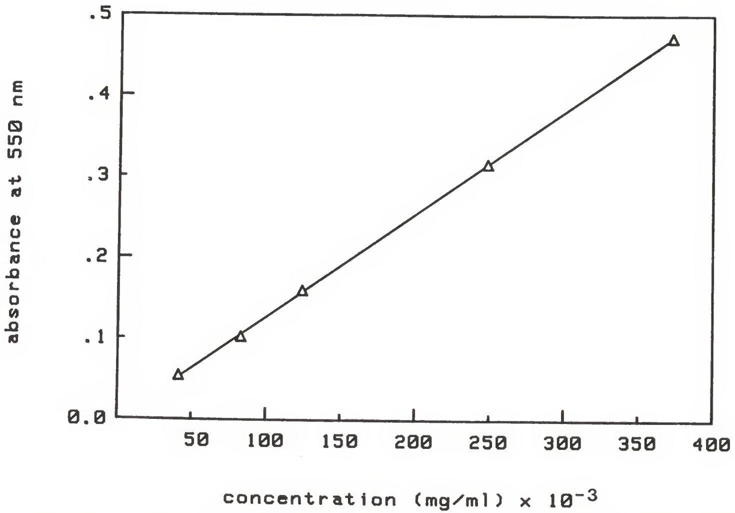


Figure 11. STM Analysis Calibration: 550 nm Absorbance vs STM Concentration.

the cloudy white mixture was adjusted from 4.32 to 5.86 by the addition of 0.1 N NaOH and mixed with a rotary mixer in the dark at 4°C for 18 hrs. The STM-PGA(14%)-BSA/MS-Q were removed from the free drug solution and the bound STM was determined as described in procedure 3.17.1. The product was white powder, 34.8 mg, 70% yield. Concentration of bound STM was 11.28 mg or 25 wt %.

3.17.3 12% Streptomycin-Polyglutamic Acid (14%)-Bovine Serum Albumin/Microspheres-Quenched and Lyophilized

A volume of 5.0 ml of the stock STM solution (51.9 mg STM) was combined with 39.7 mg of lyophilized PGA(14%)BSA/MS-Q synthesized as described in 3.12.2, (the procedure was modified in that after the last wash from the quenching stage the MS were frozen in liquid nitrogen and lyophlized). The pH of the cloudy white mixture was adjusted from 4.47 to 5.80 by the addition of 0.1 N NaOH and mixed with a rotary mixer in the dark at 4°C for 18 hrs. STM-PGA(14%)-HSA/MS-Q were washed out of the free STM solution, and concentration of the bound drug was (determined as described in procedure 3.17.1) 5.63 mg or 12 wt %. Total yield was not determined for this procedure.

3.17.4 13% Streptomycin-Polyglutamic Acid (14%)-Bovine Serum Albumin/Microspheres-Quenched (Wet)

A volume of 5.0 ml of the stock STM solution (51.9 mg STM) was combined with 3.0 ml of PGA(14%)-BSA/MS-Q (9.7 mg/ml) or 29.4 mg total MS suspended in 3.0 ml of water, in a screw cap test tube. The combination of the two solutions (STM and MS) produced a final volume of 8.0 ml. The pH of the cloudy white mixture was adjusted from 5.10 to 5.91 by the addition of 0.1 N NaOH and mixed with a rotary mixer in the dark at 4°C for 18 hrs. STM-PGA(14%)-BSA/MS-Q were washed out of the free drug solution, and concentration of bound drug was (determined as described in procedure 3.17.1) 4.39 mg or 13 wt %. Total yield was not determined in this procedure.

3.17.5 23% Streptomycin Carboxymethyldextran (12%)-Bovine Serum Albumin/Microspheres-Quenched

STM (255.2 mg) was dissolved in 25.0 of water, 5.0 ml of the clear solution (10.2 mg) was combined with 58.4 mg of CMD(12%)-BSA/MS-Q (synthesized in procedure 3.12.3) in a screw cap test tube. The pH of the cloudy white mixture was adjusted from 3.35 to 5.90 by the addition of 0.1 N NaOH and mixed with a rotary mixer at 4°C in the dark for 18 hrs. MS were washed out of the free drug solution and dehydrated and the concentration of the bound STP was determined as described in procedure 3.17.1. The product was a white powder, 60.3 mg, 85% yield. Concentration of the bound drug was 23 wt %.

3.18 In Vitro Macrophage Uptake

Dr. Edward Hoffmann of the Microbiology Department at the University of Florida was provided with a dehydrated sample of BSA/MS-U (procedure 3.7.3, average diameter 5 µm) for in vitro macrophage uptake studies.

Bovine macrophage cultures were set up by Dr. Hoffmann using the Rossi technique and allowed to mature 6 days cultured in 20% fetal calf serum with RPMI and genta. BSA/MS-U were suspended in 5.0 ml of RPMI, this equaled 3.9×10^7 MS/ml as determined with a hemocytometer. A dilution was made of the BSA/MS-U RPMI suspension so that a final concentration of 4×10^4 MS/ml was obtained. To each well containing the bovine macrophages of 0.5 ml of the MS was added and incubated at times of 30, 60, 90 and 120 mins at which time the cultures were observed with an optical microscope for MS uptake.

4. RESULTS AND DISCUSSION

4.1 Synthesis of Hydrophilic Human Serum Albumin/Microspheres

Previous methods for the synthesis of human serum albumin/microspheres (HSA/MS) involved a vegetable oil emulsion coupled with a protein denaturation process. This produced MS that were somewhat hydrophobic.

A new method was developed in this study for preparing HSA/MS which uses concentrated polymer solutions as dispersion media and achieves cross-linking of HSA by introduction of the cross-linking agent via the organic phase. Figures 12 through 14 are SEMs of HSA/MS typically produced by this method. They show a round, smooth, spherical geometry. Figure 15 clearly shows the solid internal structure of the HSA/MS. Different polymeric solutions such as polymethylmethacrylate (PMMA), Poloxamer 188, cellulose acetate butyrate (CAB) and polycarbonate were used to stabilize albumin dispersions in this process indicating the versatility of the procedure.

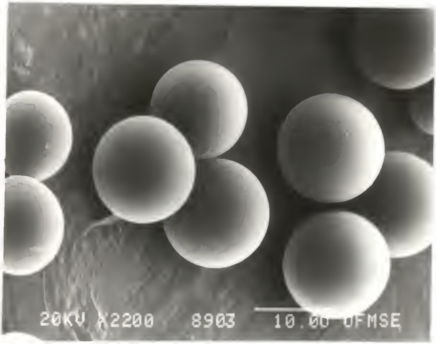


Figure 12. Scanning Electron Micrograph of HSA/MS-U, PMMA Dispersant, 10 μm Average Diameter.

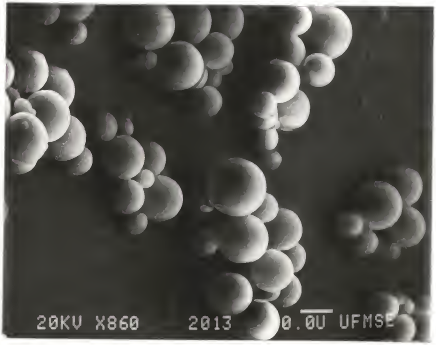


Figure 13. Scanning Electron Micrograph of HSA/MS-U, Poloxamer 188 Dispersant, 29 μm Average Diameter.

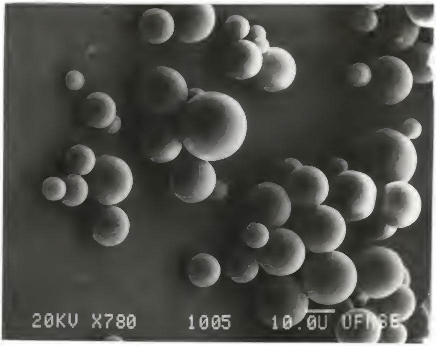


Figure 14. Scanning Electron Micrograph of HSA/MS-U, Cellulose Acetate Butyrate Disperant, 15 μm Average Diameter.

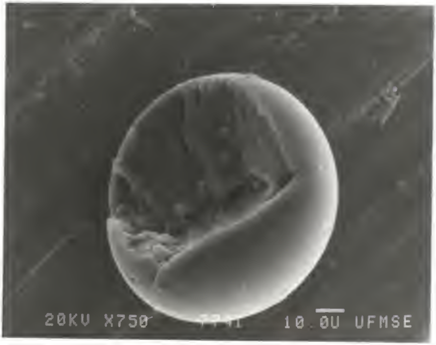


Figure 15. Scanning Electron Micrograph of 100 μm HSA/MS-U Showing Internal Structure; PMMA Dispersant.

4.2 Cross-Linking Reagents for Albumin/ Microsphere Stabilization

4.2.1 Glutaraldehyde

Glutaraldehyde was used primarily in this study for cross-linking to transform aqueous albumin dispersions into stable insoluble HSA/MS. Glutaraldehyde reacts with primary amino groups of the albumin by formation of Schiff base (imine) linkages according to the reaction scheme shown in Figure 16. In other methods for glutaraldehyde cross-linking, the commonly available 25% aqueous solution of glutaraldehyde is added to the aqueous albumin phase and requires cooling to avoid immediate cross-linking before formation of dispersions. In contrast, a technique developed in this study (procedure 3.1.1--saturating the aldehyde in organic solvents) allows glutaraldehyde to be introduced via the organic phase. The glutaraldehyde is thereby presented to the aqueous HSA dispersion from the organic phase with a resulting higher concentration of glutaraldehyde at the polymer/albumin interface. This probably produces a higher concentration of half reacted glutaraldehyde at or near the surface of the HSA/MS. This should produce a case hardening effect; i.e. a higher cross-link density at the surface due to the higher concentration of surface dialdehyde, see Figure 17.

Washed, lyophilized, or air-dried MS were readily resuspended in a variety of aqueous media such as distilled water, physiological saline, phosphate buffer, and acetate

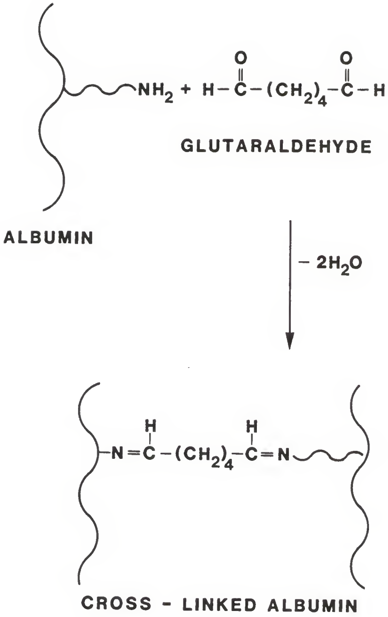
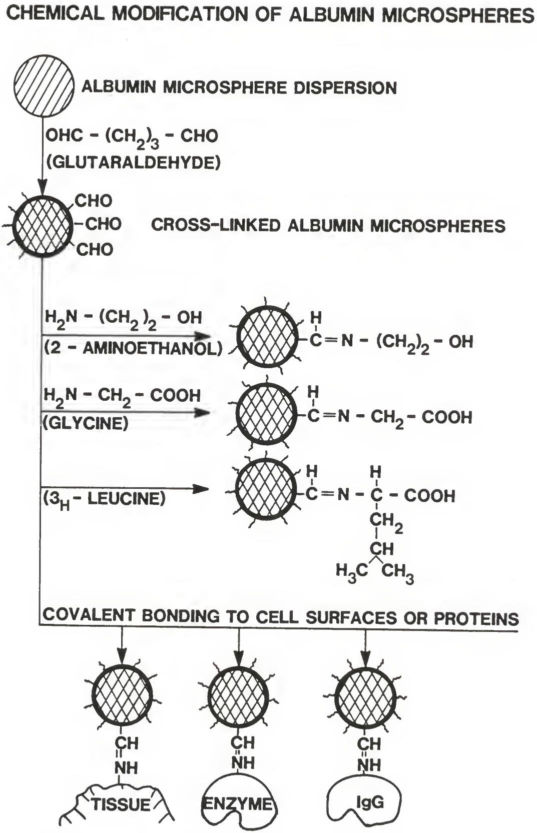


Figure 16. Reaction Scheme for the Cross-Linking of Albumin by Glutaraldehyde.



CHEMICAL MODIFICATION OF ALBUMIN MICROSPHERES

Figure 17. Schematic Representation of Albumin/MS Chemistry.

buffer. They were easily wetted and dispersed without the need for surfactants suggesting that the surface of the MS are hydrophilic. However, HSA/MS prepared by thermal denaturation in oil or by chemical cross-linking with aqueous aldehydes (incorporated at low temperatures in the aqueous albumin phase) are hydrophobic to the extent that surfactants must be used to prepare dispersions in water (61-64). This is probably a result of preferential organization of the hydrophobic regions of the albumin molecules aligning themselves at the oil interface. Thermal denaturation may enhance this effect.

There is also evidence that the hydrocarbon chains that make up vegetable oil have a strong attraction to the albumin surface and are incorporated during denaturation or cross-linking. Widder et al. (77), demonstrated that after the albumin solution emulsification in vegetable oil, undenatured or uncross-linked HSA/MS could be washed out of the vegetable oil and dehydrated with ether. The resultant free flowing white powder retained the characteristics of denatured or chemically cross-linked HSA/MS. When dispersed in a polar solvent with a surfactant, such HSA/MS retained shape for 1 to 2 hrs before dissolving. Although this phenomenon was perplexing to the authors, this temporary retention of the HSA/MS structural integrity is probably due to a hydrocarbon coating that encapsulates the aqueous albumin. Washing with ether would remove excess oil and dehydrate the protein particles. Fatty acid portions of HSA

(5-10% by weight) may facilitate this binding. The oil binding is such that numerous ether washings were unable to completely remove the hydrocarbon. Clearly, even just a monolayer coating of vegetable oil would greatly increase hydrophobicity.

This washing procedure was examined uncross-linked MS prepared by the PMMA method developed in this study (procedure 3.1.1). As the polymer dispersant was removed during the acetone wash, the uncross-linked HSA/MS lost shape and aggregated forming large macroparticles. Upon addition of water, the particles dissolved immediately indicating that no hydrophobic coating is retained using this procedure.

✓

4.2.2 Diisocyanates

Although glutaraldehyde was studied primarily, other bifunctional reagents such as the diisocyanates may also be used (88). Figures 18 and 19 are SEMS of BSA/MS that were cross-linked with 2,4-tolylene diisocyanate (TDC) and 1,6-diisocyanatohexane (DCH). These micrographs are compared with the SEM in Figure 20 showing BSA/MS that were cross-linked with glutaraldehyde. The similarity of the MS in these photographs indicate the ability of the different reagents to cross-link the ablumin. The reaction scheme for diisocyanate cross-linking is shown in Figure 21 for TDC. In procedure 3.3.1, the yield of MS cross-linked with TDC was 0.246 g, more than the starting amount of albumin. Optical micrographs taken of the washed sample showed

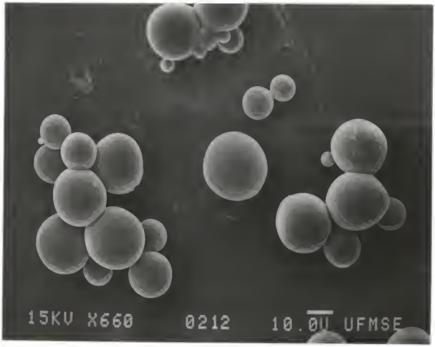


Figure 18. Scanning Electron Micrograph of BSA/MS-U, PMMA Dispersant Cross-Linked with TDC.

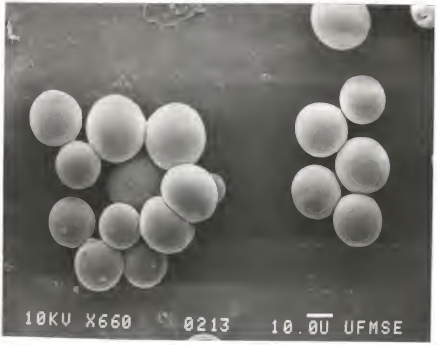


Figure 19. Scanning Electron Micrograph of BSA/MS-U, PMMA Dispersant Cross-Linked with DCH.

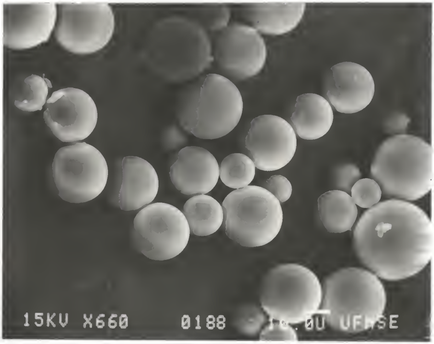


Figure 20. Scanning Electron Micrograph of BSA/MS-U, PMMA Dispersant, Cross-Linked with Glutaraldehyde.

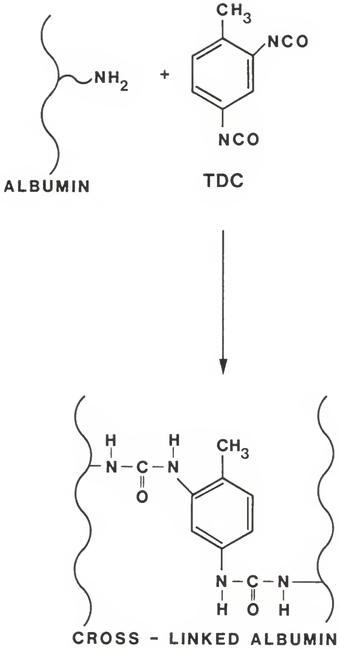


Figure 21. Reaction Scheme for the Cross-Linking of Albumin by TDC.

particles of a white crystalline material in the wash phase with the BSA/MS, this was thought to be polymerized or hydrolyzed TDC. The formation of the by-product was due to the high starting concentration of the reagent. Reducing the concentration in subsequent experiments allowed for cross-linking while eliminating this by-product. This was not observed with other cross-linking agents used in this study.

A possible advantage of the diisocyanates is their greater solubility in the organic phase allowing for direct addition to the albumin dispersion. This eliminates the ultrasonification procedure required for more water soluble reagents such as glutaraldehyde. Disadvantages of diisocyanates are their potential toxicity, especially with the aromatic TDC. In vivo toxicology studies are needed for the diisocyanate cross-linked MS to determine feasibility for use in the synthesis of HSA/MS drug carriers.

4.2.3 Aldehyde-Sucrose

A novel albumin cross-linking agent was prepared by oxidation of sucrose with periodate, the reaction scheme is shown in Figure 22. An infrared spectrum of the modified sucrose (KBr pellet) is shown in Figure 23. The peak at 1600 cm^{-2} corresponds to an aldehyde carbonyl stretch. No such peak exist in the infrared spectrum of sucrose (Figure 24). The aldehyde-sucrose was used to cross-link the MS. SEM and optical micrographs of the BSA/MS cross-linked with aldehyde-sucrose are shown in Figure 25 and 26.

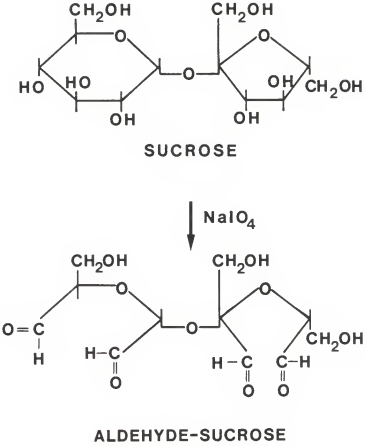


Figure 22. Reaction Scheme for the Synthesis of Aldehyde-Sucrose.

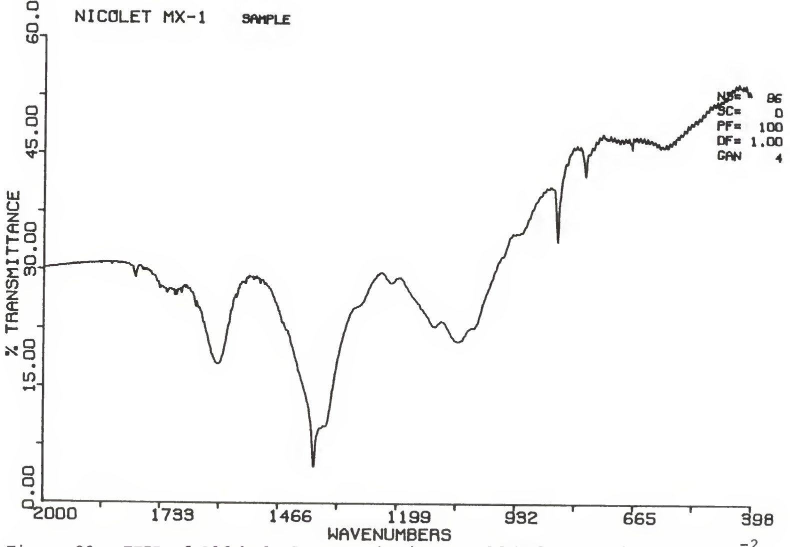


Figure 23. FTIR of Aldehyde-Sucrose Showing an Aldehyde Stretch at 1600 cm^{-2}.

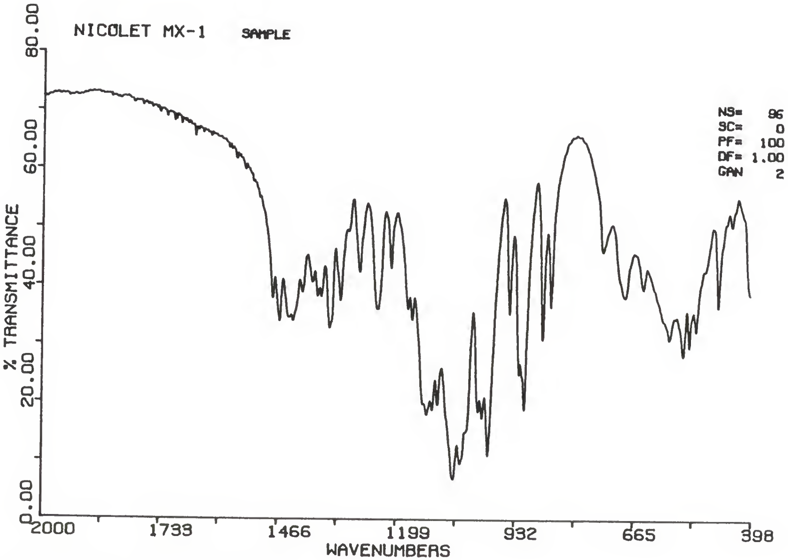


Figure 24. FTIR of Sucrose.

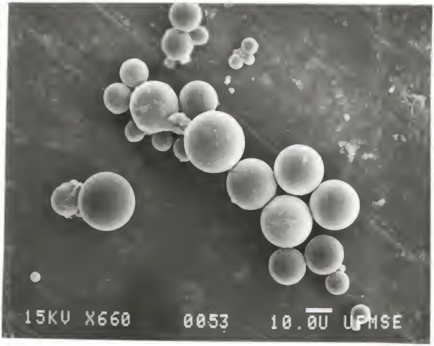


Figure 25. Scanning Electron Micrograph of BSA/MS-U, PMMA Dispersant, Cross-Linked with Aldehyde-Sucrose.

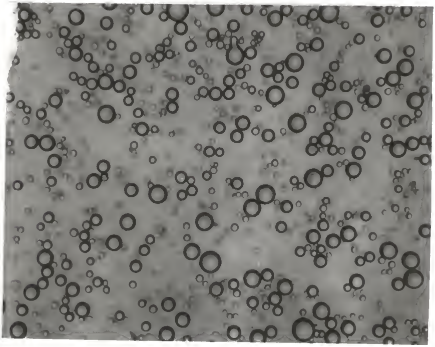


Figure 26. Optical Micrograph (100 X) of BSA/MS-U, PMMA Dispersant, Cross-Linked with Aldehyde-Sucrose.

Cross-linking time was longer for the aldehyde-sucrose than with either glutaraldehyde or the diisocyanates. This opens the possibility of adding the aldehyde-sucrose to the aqueous albumin phase before dispersion in the polymer solution while at r.t. (without the cooling needed for faster-reacting glutaraldehyde). Aldehyde-sucrose may provide an inexpensive more bioacceptable substitute for glutaraldehyde or diisocyanates.

4.3 Effect of Polymer Dispersant Concentration

Concentrated polymer solutions afforded excellent stabilization for aqueous albumin dispersion. Optical photomicrographs (Figures 27 and 28) show a polymer coating (Poloxamer 188) surrounding the HSA/MS dispersion in the aqueous phase. This polymer coating or microencapsulation apparently affords dispersion stability and prevents MS aggregation before and during gluataraldehyde cross-linking. √

Data presented in Table 2 indicate that a polymer concentration of at least 20 wt % was needed to stabilize dispersions at short dispersion times (1 min). When smaller MS are desired (requiring longer dispersion times or higher energy input) higher concentrations of polymer are needed. This is also shown in Table 2. The concentration of the polymer dispersant solution was important. Concentrations below 25 wt % were not sufficient to prevent coagulation when small MS (less than 10 μm) were desired, and concentrations above 30 wt % retarded removal of the √

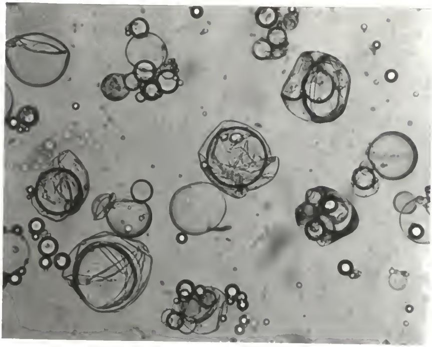


Figure 27. Optical Micrograph (200 X) of Stabilized HSA/MS-U Showing the Surrounding Polymer Coating, MS in Aqueous Wash Phase, Poloxamer 188 Dispersant.

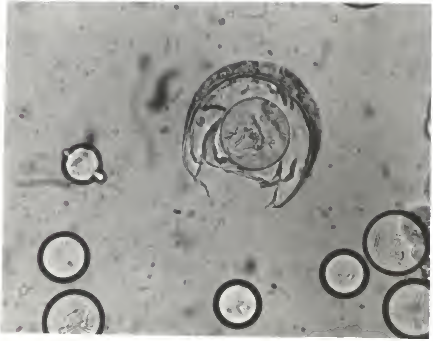


Figure 28. Optical Micrograph (200 X), Poloxamer 188 Coated MS, as in Figure 26, Showing Ruptured Coating During Wash Phase.

polymer. Polymer MW was also important. Low MW PMMA (intrinsic viscosity 0.4) was unsatisfactory at concentrations of 20-30 wt %, since aggregation occurred.

These observations are in agreement with the work of Heller (89) on the stabilization of dispersed aqueous gold sols in polyethylene glycol. Heller demonstrated that microparticle stabilization was due to adsorption of macromolecules at the surface of the microparticles ("steric stabilization") making it impossible for them to approach each other closer than some critical distance. This inhibited the inelastic collisions responsible for aggregation or destabilization of microparticles. It was also shown that the steric stabilization increased with increasing molecular weight and polymer concentration to a constant value. Heller's experiments were performed with solid microparticles that had a net negative charge. It was assumed that the polymer adsorbed to the surface in some reversible manner. To displace the polymer from a microparticle, the washing solvent must have a higher affinity for the particle surface than the adsorbed macromolecules. The procedure described in this study appears quite analogous to the steric stabilization reported for colloids.

Solvents chosen for each polymer were also critical for dispersion stability during MS synthesis. Poloxamer 188 and PMMA were both soluble in toluene/chloroform mixtures, but Poloxamer 188 would not stabilize the dispersion in this two solvent mixture although PMMA was satisfactory in toluene/chloroform. However, when Poloxamer 188 was dissolved in only chloroform, satisfactory stabilization was achieved (procedure 3.2.1). Similarly enough, cellulose acetate butyrate (CAB) is soluble in both dioxane and ethylene dichloride, but only ethylene dichloride was satisfactory for preparing HSA/MS. Although the reason for these critical interactions between solvent and polymer have not been fully explored, they are probably due to variations in solvation. More fully solvated polymer chains would be in more chain extended conformation thereby optimizing the steric stabilization effect as depicted in Figure 29.

4.4. Microsphere Size Distribution

The size distribution of the HSA/MS could be easily controlled in this synthesis. Figures 30 and 31 show the typical size distributions of HSA/MS as a function of dispersion time using the vortex apparatus. PMMA was used as a dispersant for the HSA/MS in Figure 30 and Poloxamer 188 for those in Figure 31. For the dispersion times studied (1-8 mins) the HSA/MS had an average size distribution from 7-19 μm as measured with a Coulter Counter. Average diameters determined from the Coulter Counter were in close agreement with data obtained from SEM measurements of the same samples, (7-22 μm). SEM size distribution as a function of power input is shown in Figure 32. The

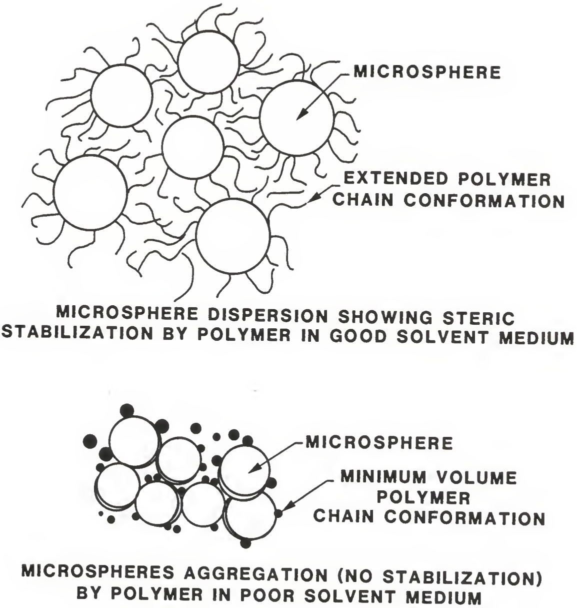

MICROSPHERE
EXTENDED POLYMER CHAIN CONFORMATION
MICROSPHERE DISPERSION SHOWING STERIC STABILIZATION BY POLYMER IN GOOD SOLVENT MEDIUM
MICROSPHERE
MINIMUM VOLUME POLYMER CHAIN CONFORMATION
MICROSPHERES AGGREGATION (NO STABILIZATION) BY POLYMER IN POOR SOLVENT MEDIUM

Figure 29. Schematic Representation of Steric Stabilization.

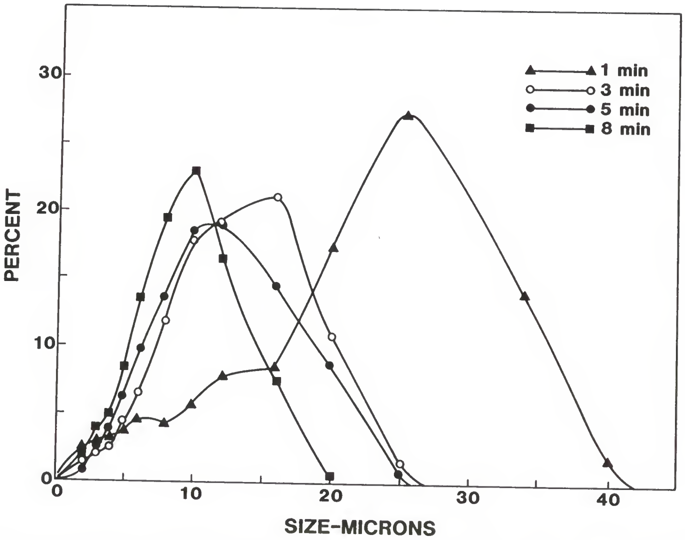


Figure 30. Coulter Counter Size Distribution of HSA/MS-U using PMMA as Dispersant, Constant Power Input at Setting 9, Time of Dispersion Varied.

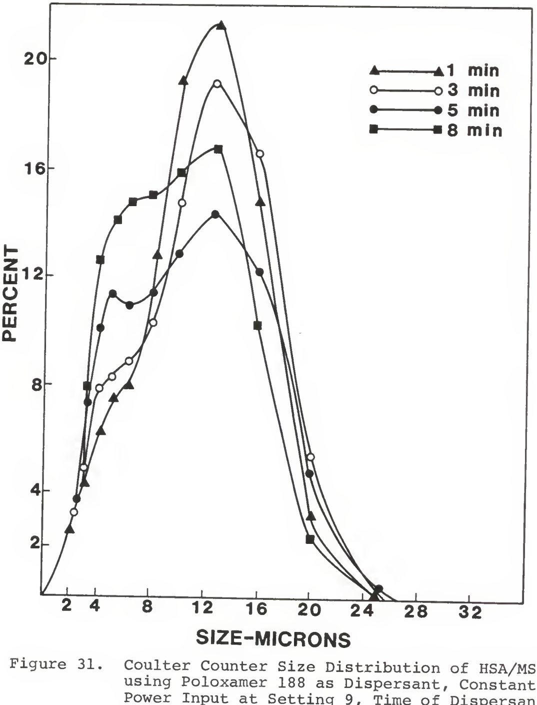


Figure 31. Coulter Counter Size Distribution of HSA/MS-U using Poloxamer 188 as Dispersant, Constant Power Input at Setting 9, Time of Dispersant Varied.

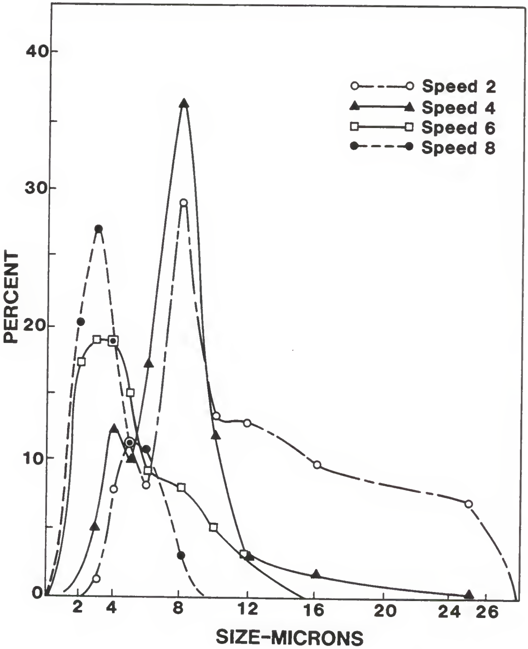


Figure 32. Scanning Electron Micrograph Measurements of Size Distributions for HSA/MS-U using PMMA as Dispersant, Constant Disperson Time 10 mins, with Power Input Varied.

dispersion energy input in the vortex genie was varied from 2 to 8 and produced an average size distribution of 4-9 μm.

The size of the MS was directly related to power input and dispersion time. Increasing either the power input or dispersion time decreased the size distribution of the HSA/MS.

4.5 Synthesis of Sub-Micron Microspheres

The synthesis of sub-micron HSA/MS was possible by a modification of the CAB process described in procedure 3.1.3. Figure 33 shows the size distribution of HSA/MS as a function of mixing time and energy input. At an energy level (speed setting 5.5 on the tissue homogenizer) and 5 mins mixing time, size of the HSA/MS ranged from 0.75-4.5 μm. However, increasing the energy level (6.5) and mixing time (10 mins) a HSA/MS size distribution of 0.2-1.5 μm with an average diameter of 0.8 μm was obtained. SEM of the sub-micron HSA/MS are shown in Figures 34. The size distributions were obtained using the SEM because the electronic particle counter is not satisfactory at sub-micron particle size.

Using the vortexing apparatus to disperse the aqueous albumin in polymer solutions was convenient, simple and readily produced HSA/MS size distributions of 3 to 150 μm. However, when sub-micron HSA/MS are required, the vortexing apparatus was inadequate because of limited dispersion power

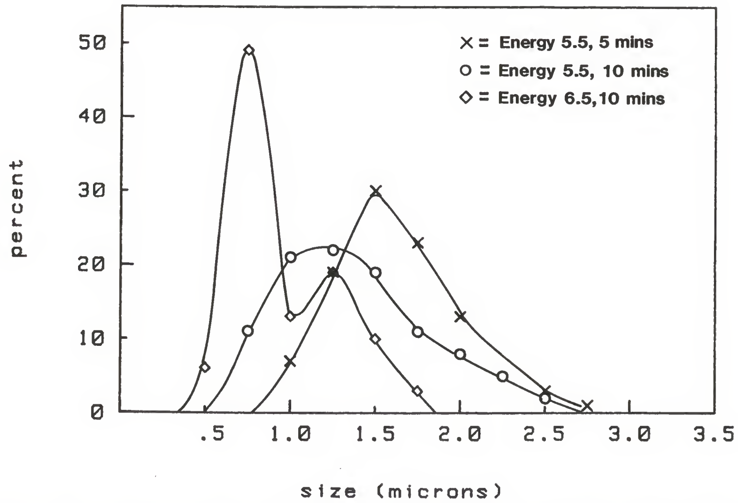


Figure 33. Scanning Electron Micrograph Measurements of Sub-Micron Size Distributions for HSA/MS-U as a Function of Time and Energy Input in Cellulose Acetate Butyrate Dispersion.

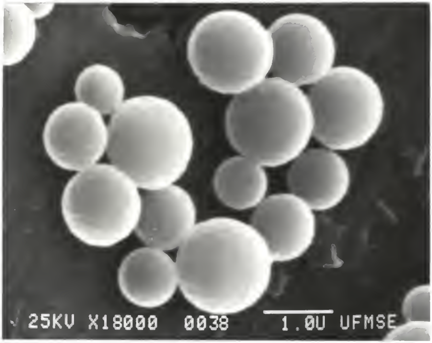


Figure 34. Scanning Electron Micrograph of HSA/MS-U, Cellulose Acetate Butyrate Dispersant, 0.8 μm Average Diameter, Homogenizer Setting 6.5, Mixing Time 10 mins.

and extremely long vortexing times required. Good dispersion for sub-micron HSA/MS were easily obtained with the tissue homogenizer, which is designed for high shear rates to emulsifing solid tissue in short times.

As discussed (Section 4.3), stabilization of microdispersions in polymeric solutions may be related to the ability of the macromoleculer solution to prevent inelastic collisions between microparticles. For smaller MS, the number per given sample volume increases as does the surface area available for collision. By increasing the volume of the polymer solutions (decreasing the microdispersion concentration) it was possible to stabilize the sub-micron HSA/MS before and during the cross-linking procedure. All attempts to produce sub-micron MS using smaller volumes (high dispersion concentrations) were unsuccessful. CAB was used because of the lower concentration of the polymer that is needed for stabilization but other polymers such as PMMA may also work.

The synthesis of sub-micron HSA/MS (less than 1 μm) is potentially very important because it would allow i.v. administration of HSA/MS preparations. Studies have shown that HSA/MS above 4 μm tend to be trapped in the capillary bed of the lungs (32). This complicates drug delivery unless the lungs are the primary target. Sub-micron HSA/MS would easily clear the lung capillaries for systemic therapy. Larger HSA/MS (greater than 25 μm) are used for soft tissue injections (i.m., i.p.), were size enables physical immobilization.

4.6 Hydration as a Function of Cross-Linked Density

The cross-link density of the ablumin/MS was controllable by varying the concentration of the cross-linking agent. This was measured by size increase of MS and the weight of water uptake due to hydration. Figure 35 relates concentration of cross-linking reagents to water uptake of BSA/MS. MS swelling increased at lower concentrations of reagent (reduced cross-link density). Optical micrographs in Figures 36 through 41 show BSA/MS cross-linked with varying amounts of aldehyde-sucrose. Each sample is shown in its dehydrated and hydrated state. For the three preparations, as the concentration of aldehyde-sucrose was reduced, the average diameter of dehydrated MS remained constant. However, when hydrated, MS size increased at low cross-link densities (decreasing concentrations of aldehyde-sucrose).

The curves in Figure 35 show that TDC, glutaraldehyde and aldehyde-sucrose have a higher reactivity as compared to DCH. To obtain this comparison, a dilution factor was first calculated. This factor is required because the diisocyanates are toluene soluble so that they were added directly to the organic phase. Glutaraldehyde and aldehyde-sucrose on the other hand had to be put into a toluene saturated solution by ultrasonification. For example, in a typical

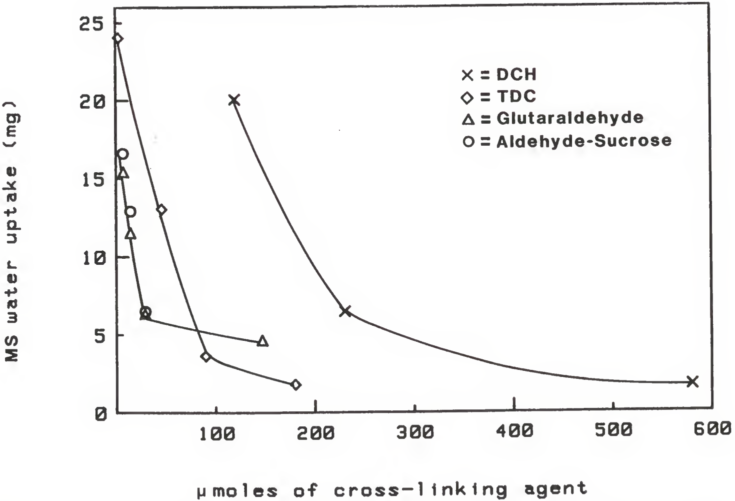


Figure 35. Concentration of Cross-Linking Agent vs Amount of Water Uptake/mg of BSA/MS-U.

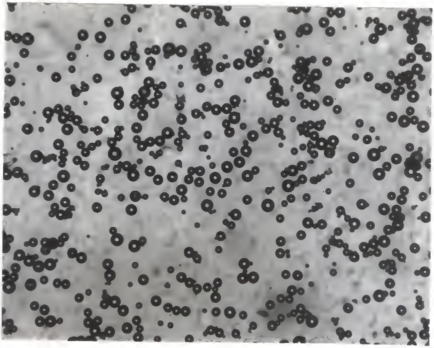


Figure 36. Optical Micrograph (100 X) of Dehydrated BSA/MS-U, Average Diameter 19 µm, PMMA Dispersant, Cross-Linked with 0.5 mmoles of Aldehyde-Sucrose.

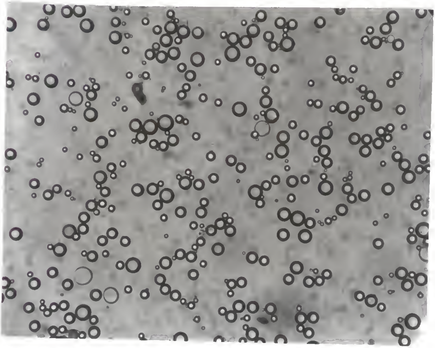


Figure 37. Optical Micrograph (100 X) of Hydrated BSA/MS-U, Average Diameter 20 µm, PMMA Dispersant, Cross-Linked with 0.5 mmoles of Aldehyde-Sucrose.

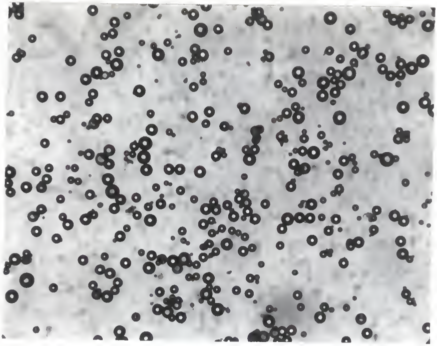


Figure 38. Optical Micrograph (100 X) of Dehydrated BSA/MS-U, Average Diameter 19 μm, PMMA Dispersant, Cross-Linked with 0.25 mmoles of Aldehyde-Sucrose.

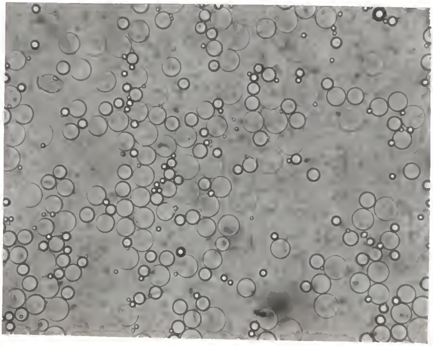


Figure 39. Optical Micrograph (100 X) of Hydrated BSA/MS-U, Average Diameter 27 μm, PMMA Dispersant, Cross-Linked with 0.25 mmoles of Aldehyde-Sucrose.

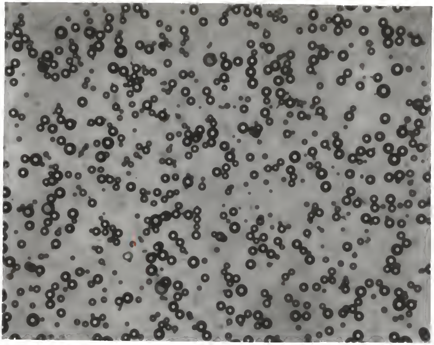


Figure 40. Optical Micrograph (100 X) of Dehydrated BSA/MS-U, Average Diameter 18 μm, PMMA Dispersant, Cross-Linked with 0.125 mmoles of Aldehyde-Sucrose.

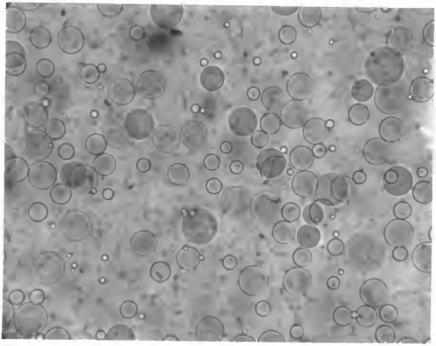


Figure 41. Optical Micrograph (100 X) of Dehydrated BSA/MS-U, Average Diameter 40 μm, PMMA Dispersant, Cross-Linked with 0.125 mmoles of Aldehyde-Sucrose.

preparation for a starting concentration of 2.5 mmoles glutaraldehyde after toluene saturation (procedure 3.1.1), the toluene contained 0.14 mmoles glutaraldehyde as determined by a 3-methyl-2-benzothialzoline hydrazone method (90). Correcting the other starting glutaraldehyde and aldehyde-sucrose concentrations by this 17 X dilution and comparing results with TDC shows that the concentration dependence of the cross-linking agents vs water uptake (i.e. corss-link desnity) are relatively close.

4.7 Chemical Modification of Albumin/Microspheres Cross-Linked with Glutaraldehyde

As stated, addition of the gluataraldehyde to the aqueous albumin micro-dispersion may produce a high cross-link density at or near the surface of the MS. This would also produce a high concentration of half-reacted glutaraldehyde (free CHO groups) at or near the surface of the MS. These aldehyde groups can be used as a chemical handle for further surface and bulk modification of MS. Because primary amines readily react with CHO groups, the aldehyde groups can be quenched or "capped" with molecules containing primary amino groups such as 2-aminoethanol or glycine (Figure 17). This increases the hydroxyl and carboxyl content of the MS which further enhances the anionicity or hydrophilicity. The high concentration of aldehyde groups also allows other chemical modifications involving large molecules such as enzymes, antibodies, proteins and amino-functional drugs. As suggested in

Figure 17, such changes in surface functionality may be used to enhance tissue immobilization by covalent binding or for specific tissue targeting using biospecific affinity ligands (e.g. tumor-specific antibodies or antigens).

The binding of molecules (containing primary amino groups) to aldehyde groups of unquenched MS demonstrates the versatility of this simple procedure to allow for surface modification which requires only the addition of the compound to MS solution.

4.8 Measurement of Reactive Aldehyde Groups

The number of reactive aldehyde groups was measured for a preparation of HSA/MS (quenched and unquenched) that had an average diameter of 10 m. The concentration of tritiated leucine bound to reactive aldehyde groups is shown in Table 10. By subtracting the amount of adsorbed [^{3}H] leucine (measured using quenched MS) from the amount adsorbed and covalently bound to the unquenched HSA/MS, it was determined that 4.0×10^{-2} moles of leucine was bound to aldehyde groups in 1.0 ml of water containing 8.0 mg of HSA/MS. Electronic particle analysis established that there were approximately 1.0×10^{10} MS per 10 ml in a 10μm diameter preparation. Using the equation

$$N_{CHO} = \frac{a \times b}{C}$$

where N = the number of aldehyde groups, a = moles of bound leucine/1.0 ml of HSA/MS suspension, b = Avogadro's number and c = number of MS/ml, 2.4×10^7 reactive CHO groups per MS was calculated based on one leucine molecule covalently bound per reactive group. From the data of Ikada et al. (91), 1.1 μg of an HSA monolayer would cover a 1.0 cm area. Using $S = \pi d^2$, for the surface area of a sphere, the surface area for 10 μm diameter MS is 3.14×10^{-10} m^2. Since 1.1 μg of HSA covers 1.0 cm^2, 1.1×10^{-2} g of HSA would cover 1.0 m^2 with a monolayer of protein. Multiplying surface area of a 10 μm MS by the amount of protein needed to cover a m^2 area equals 3.45×10^{-12} g of albumin. Using the equation

$$N_{HSA} = \frac{AM}{MW} \times b$$

where N = the number of HSA molecules on the surface of a 10 μm MS and AM = the amount of albumin in g on the surface, this gives

$$NHSA = \frac{3.45 \times 10^{-12} \text{ g}}{69{,}000} \times 6.02 \times 10^{23}$$

$$= 3.01 \times 10^{7} \text{ HSA moleclues}$$

Dividing the number of aldehyde groups by the number of HSA molecules, it was determined that there are 79 CHO/100 HSA molecules. This is a maximum value, since a fraction of CHO groups must also be present within the MS. As the average diameter of the HSA/MS was reduced (20, 10, and 3 μm), it was found that the amount of bound leucine remained relatively constant (Table 11). This indicated that a small molecule like leucine binds not only at the surface but also to aldehyde groups within the MS.

However, using tritiated Concanvalin A (Con-A), a protein molecule 100 times larger than leucine, it was found that as the MS size decreased (30, 12, and 5 μm) the amount of bound Con-A increased (Table 12). This indicated that Con-A binding was surface area dependent. The larger molecule apparently reacts at the surface and does not penetrate into the HSA/MS. In this experiment (3.11.3), it was also found that at a size of 30 μm, there was no difference in the amount of bound Con-A comparing the quenched and unquenched MS. This could be due to the natural affinity of the Con-A for the albumin. As the surface area increased (i.e. smaller MS), the fraction of covalently bound Con-A may have increased. In future studies, this question of Con-A binding may be clarified by washing the MS with a 0.1 molar solution of glucose after coupling. Con-A has a high affinity for glucose. Complexed but not covalently bound Con-A would thereby be removed.

4.9 Determination of Residual PMMA on the Surface of Cross-Linked Microspheres by Electron Scattering Chemical Analysis (ESCA)

The surface of HSA/MS was analyzed to a depth of 25-50 Å using ESCA to detect possible residual polymer (PMMA) on the MS surface after washing. A depth of 50 Å represents 0.05% of the total volume in a 10 μm MS. Table 13 gives the (N/C), (O/C), and (N/O) ratios for HSA/MS at various stages of the acetone wash (procedure 3.12.2) for HSA cross-linked with glutaraldehyde (but not polymer dispersed). The data indicate that as the number of acetone washings increases, the nitrogen concentration detected at the MS surface also increases resulting in higher N/C and N/O ratios.

Since PMMA contains C and O, (hydrogen is not detected with ESCA) and albumin contains C, O, N, and some S, the N/C and N/O ratios afford a means of identifying HSA. If the MS were coated with PMMA to a 50 Å depth, little or no N would be detected. As the depth of PMMA is decreased during the acetone washing, the amount of N analyzed would increase accordingly. If the PMMA were completely removed, the N/C and N/O would be equal to the albumin control of HSA cross-linked with glutaraldehyde. The data in Table 13 indicate that after 8 acetone washes, the PMMA was completely removed from the MS surface.

4.10 Capillary Wetting

A technique was sought that would enable measurement of surface energy of the MS produced in this study for comparison with hydrophobic MS synthesized using the vegetable oil method. Different methods were explored (such as contact angle measurements, etc.) but due to the small particle size such methods were not feasible.

However, a capillary method for surface energy using tube wetting was developed by Richards and Carven (92). Their technique involves the placement of a capillary tube vertically in water to a known depth. The height of the water rise inside the tube then depends on the surface tension of the capillary tube material. The more hydrophilic or hydrophobic the material, the higher or lower the rise of water (height of meniscus inside the tube). From the height measurements, the surface energy of the material can be determined using appropriate equations and correction terms reviewed elsewhere (92). It was thought that a modification of this capillary technique (i.e. packing the tubes with MS) might allow quantitative measurements of surface energy (wetting) for the HSA/MS.

A method was developed to pack pasteur pipets in such a way that reproducible results for capillary rise could be obtained. This technique was used for wetting measurements for HSA/MS-U (3 mm), HSA/MS-Q (27 mm) and hydrophobic MS (0.1 mm). Figure 42, compares the water rise vs time for the HSA/MS preparations that were studied.

Naively, it was first thought that the data obtained could be used to calculate surface energy. However, packing microparticulate HSA/MS in a capillary tube produces an additional capillary effect because each MS itself contains hundreds of microcapillaries due to the porous structure. This and other critical parameters such as size distribution, and packing factors further complicate the analysis. Although this technique was not studied in sufficient detail to assign surface energies to MS, it did enable quantitative comparisons of hydrophilicity for

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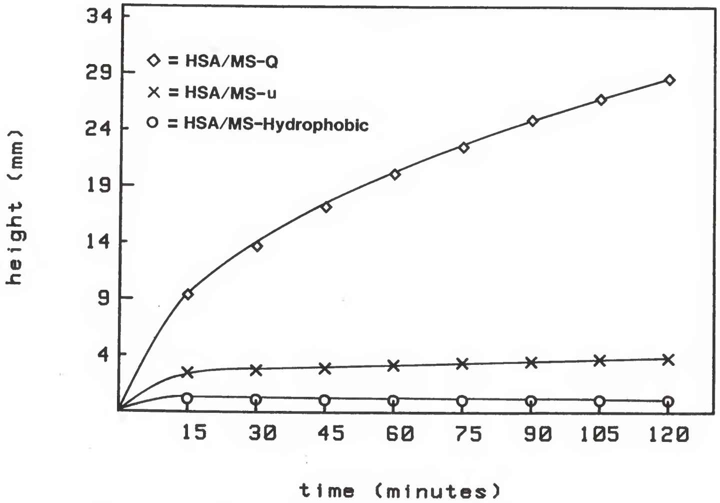


Figure 42. Capillary Rise (MS Wetting) vs Time.

HSA/MS synthesized by different procedures. Studies with soils using a similar procedure have also demonstrated the utility of the method for quantitative comparisons between samples and indicated the problems in obtaining surface energies for soil particles (93).

The largest capillary rise (wetting) was obtained for HSA/MS-Q quenched with glycine. This indicates that by increasing the carboxyl content of the MS there was an increase in the hydrophilicity. As expected, no wetting was observed for the hydrophobic HSA/MS produced by the vegetable oil method.

Further study is required to refine the capillary technique to a point where surface energy measurements can be made for HSA/MS and may involve the use of standards such as MS made of materials with known surface energies.

4.11 Polyanionic Modification of Albumin Microspheres by Addition of Polyglutamic Acid

The HSA/MS produced in this study have a slightly cationic charge due to the basic amino acid residues that in albumin. By incorporation of polyglutamic acid (PGA) into the albumin matrix, it was thought that MS could be produced with a net negative charge (due to the high carboxyl group content of PGA). This was considered important because of previous work by Terry (38) who demonstrated that anionic macromolecules such as PGA could bind high concentrations of basic drugs by the formation of a salt complex.

Thus, adding PGA to the MS, high drug loadings (20-40%) might be obtained.

It was found that PGA-HSA/MS could be produced containing up to 20 wt % of the synthetic polypeptide. Figure 43 is a SEM of PGA(20%)-HSA/MS showing that MS were formed with incorporated PGA. When concentrations of 25% or more of the 60,000 MW PGA were added to the albumin, it interfered with cross-linking MS to the extent that PGA(25%)-HSA/MS broke apart after synthesis. At these high concentrations, PGA may produce boundary areas inside the MS that are uncross-linked.

4.12 Polyanionic Modification of Albumin/Microspheres by Addition of Carboxymethyldextran

As described in Section 4.11, incorporation of PGA into HSA/MS increased anionicity. For drug delivery applications involving large scale preparation of anionic MS (e.g. treatment of Brucellosis in cattle), the use of PGA might be limited because of high cost. One alternative to PGA is carboxymethyldextran (CMD). The anionic dextran is less expensive and permits addition of a high concentration of carboxyl groups to the albumin/MS. An SEM for CMD(15%)-BSA/MS is shown in Figure 44 and demonstrates that BSA/MS could be produced with the modified dextran. The optical micrograph in Figure 45 shows that the CMD-BSA/MS have a two phase structure, (compared with BSA/MS in Figure 46). The dark areas of the MS are probably due to the difference in refractive index between the albumin and dextran.

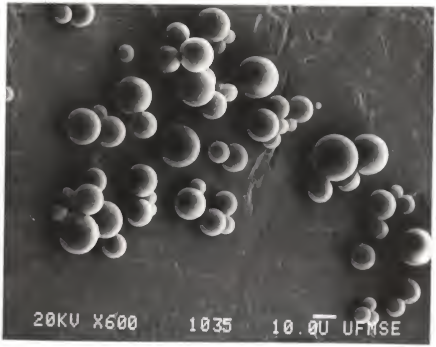


Figure 43. Scanning Electron Micrograph of PGA(20%)-HSA/MS-U, PMMA Dispersant, 19 μm Average Diameter.

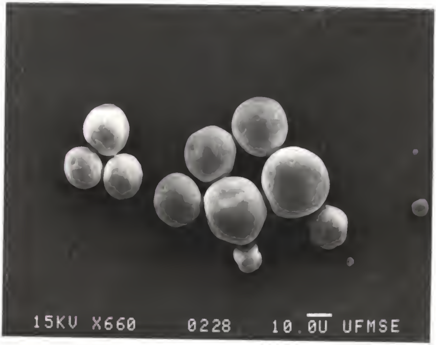


Figure 44. Scanning Electron Micrograph of CMD(15%)-BSA/MS-U, PMMA Dispersant

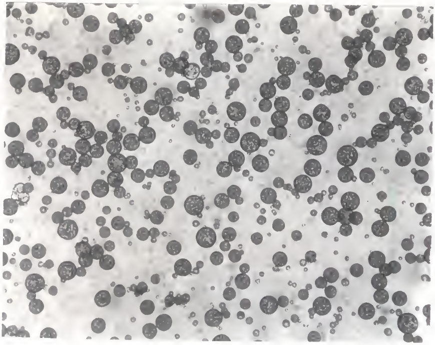


Figure 45. Optical Micrograph (100 X) of CMD(15%)-BSA/MS-U, PMMA Dispersant

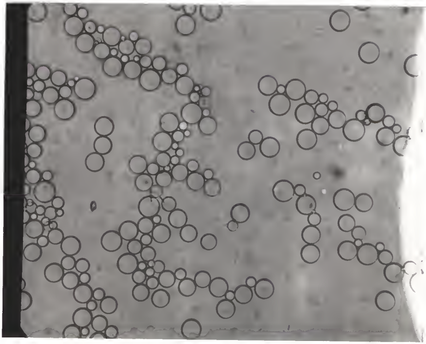


Figure 46. Optical Micrograph (100 X) of BSA/MS-U, PMMA Dispersant

4.13 Polycationic Modification of Albumin/ Microspheres by Addition of Polylysine

Polylysine (PLY) is a cationic polypeptide that consists of repeating units of an amino-functional amino acid. PLY was readily incorporated into HSA/MS at various concentrations. It was also possible to make MS entirely composed of PLY. Glutaraldehyde was used to cross-link the PLY molecules in the same manner as albumin. PLY/MS may be useful for complexing acidic drugs by formation of salt complexes. However, it has been well established that PLY in the MW range of 10,000 to 70,000 is extremely toxic to animals, but MW below 10,000 are relatively non-toxic in moderate amounts (94). The toxicity of such MS requires investigation. The SEM in Figure 47 shows PLY/MS produced using the PMMA method (3.2.6).

4.14 Albumin/Microspheres for Veterinary Applications

The use of foreign proteins for drug carriers in mammals may introduce serious immune response problems. It may be advantageous to synthesize MS from albumin of the particular species or individual to be treated to avoid undesired immune response. This may also be important in animal treatment. Figures 48, 49 and 50 show optical micrographs of MS synthesized from bovine, dog and rabbit albumin. The MS are readily formed and are similar to MS produced from human albumin. This further demonstrates the versatility of the procedure. The ability to synthesis MS

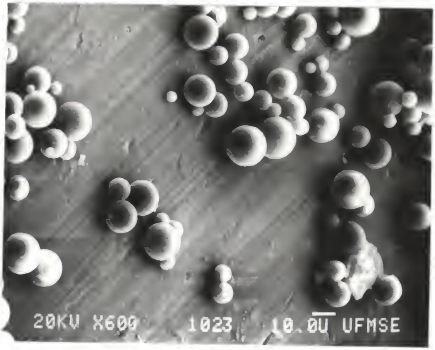


Figure 47. Scanning Electron Micrograph of PLY/MS-U, PMMA Dispersant

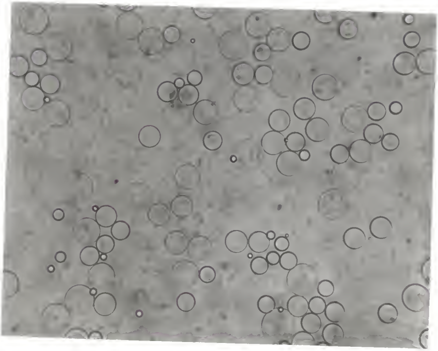


Figure 48. Optical Micrograph (200 X) of BSA/MS-U, PMMA Dispersant, Average Diameter 28 μm

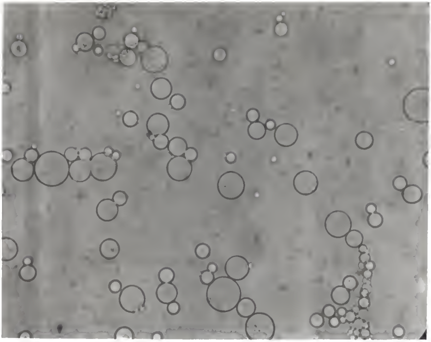


Figure 49. Optical Micrograph (200 X) of Dog Serum Albumin/MS-U, PMMA Dispersant, Average Diameter 14 μm.

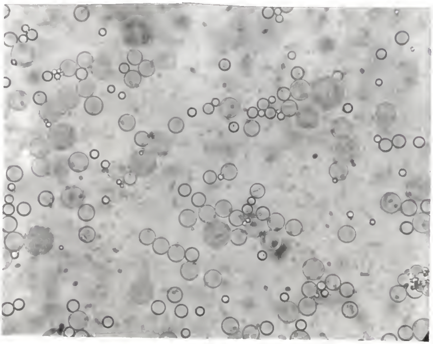


Figure 50. Optical Micrograph (200 X) of Rabbit Serum Albumin/MS-U, PMMA Dispersant, Average Diameter 13 μm.

from other mammalian protein may be beneficial for veterinary applications where MS containing therapeutic agents are required.

4.15 Drug Preparations for Albumin/Microspheres

The hydrophilic albumin/microspheres prepared in this study were investigated for drug uptake and mechanisms of drug binding. Drug release properties for the MS were also studied using in vitro (dynamic flow and fixed volume). In vivo toxicity, immobilization and degradation studies were also conducted with CD-1 mice. Therapeutic agents examined for MS binding included adriamycin (AD) and bleomycin (BLM) two anti-cancer drugs that have broad spectrum anti-tumor activity. These drugs have already demonstrated increased efficacy when administered by i.t. injection (42,43). Gentamycin (GMC) and streptomycin (STM), two broad spectrum antibiotics, were also studied for HSA/MS binding properties to the MS and their subsequent drug release. MS containing either GMC or STM may be useful for the treatment of bacterial infections such as P. Aeruginosa of the eye and brucellosis infections in cattle (79,81).

It should also be emphasized that the hydrophilicity of HSA/MS developed in this study, allows for the incorporation of therapeutic agents to the albumin/MS after synthesis. This facilitates (1) higher drug loadings, and (2) binding of chemically sensitive drugs that might may be effected by thermal or chemcical conditions of conventional MS synthesis.

4.15.1 Adriamycin-Albumin/Microspheres

HSA/MS were readily prepared containing up to 18 wt % of the anti-tumor drug adriamycin (AD) by adding MS to aqueous solutions of AD. The binding of AD to HSA/MS involved two mechanisms: (1) the covalent attachment of the primary amino group of AD to reactive aldehyde groups of HSA/MS-U, and (2) physical binding of AD to HSA/MS-Q.

Terry (38) demonstrated that PGA readily formed stoichiometric ionic salts with basic drugs such as AD. This was due to interaction between the anionic PGA carboxyl groups and the cationic primary amino group located on the daunosamine ring of AD, (Figure 51). Terry also found that the PGA-AD salt would dissociate in aqueous electrolytes to release AD. The addition of PGA to the HSA/MS made possible the preparation of AD-PGA-HSA/MS containing up to 45 wt % of the drug through the formation of this AD-salt complex. This is demonstrated in the comparison of binding data for AD in Table 42 for (HSA/MS-U and PGA-HSA/MS-U) and Table 43 for (HSA/MS-Q and PGA-HSA/MS-Q). The data show that as the concentration of PGA increases in the MS, the concentration of bound AD also increases. These data also show that the amount of bound AD is higher for the MS-U than the MS-Q at comparable concentrations of PGA. This is attributed to the increased amount of AD that can be covalently bound to

Table 42

Amount of Bound AD for Unquenched HSA/MS and PGA-HSA-MS
(Preparations 3.13.1 - 3.13.4)

Amount of Added PGA	Weight % Bound AD
0	18
12	33
16	39
22	46

Table 43

Amount of Bound AD for Quenched HSA/MS and PGA-HSA-MS
(Preparations 3.14.5 - 3.13.8)

Amount of Added PGA	Weight % Bound AD
0	18
11	21
15	25
19	33

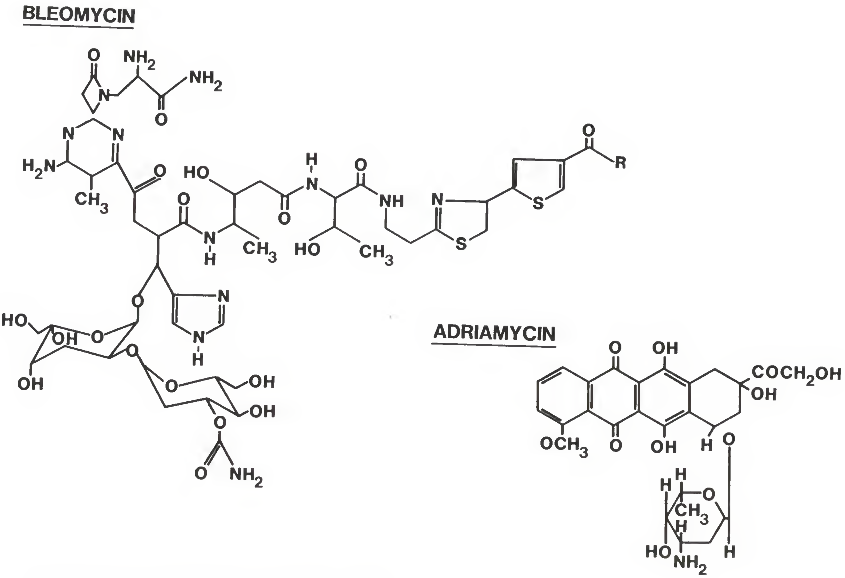


Figure 51. Structures of BLM and AD.

HSA/MS-U as compared to the physically associated AD in HSA/MS-Q.

The ionic AD-salt complex was shown to be pH sensitive. Figure 52 shows the increase in AD binding to PGA-HSA/MS-Q as a function of increasing pH in the range of 3-7 for PGA(15%)-HSA/MS-Q, (Table 29). The pH for maximum binding was found about 6.0 and corresponded to 32 wt % bound AD for PGA(15%)-HSA/MS-Q. The data suggest that for high efficiency of AD binding to MS the pH should be controlled throughout the process at a pH of 6.0.

To determine if AD penetrated the interior of the MS during the binding process or was primarily surface bound, AD-HSA/MS were mounted in epoxy and serial section with a ultra-microtome. The sections were mounted on TEM grids and examined with an optical microscope. The red chromophore of AD could be seen throughout the slices and this indicated that the drug had diffused into the interior of the HSA/MS.

In vitro AD release. AD release rates from AD-HSA/MS and AD-PGA-HSA/MS were evaluated using a dynamic flow column method described in Section 3.14.1. The release rates for AD-HSA/MS and AD-PGA-HSA/MS were found to be readily controlled and due to the three distinct binding mechanisms: (1) slow release--hydrolytic degradation of covalent bonds, (2) medium release--dissociation of the drug salt complex, and (3) fast release--desorption of physically associated drug.

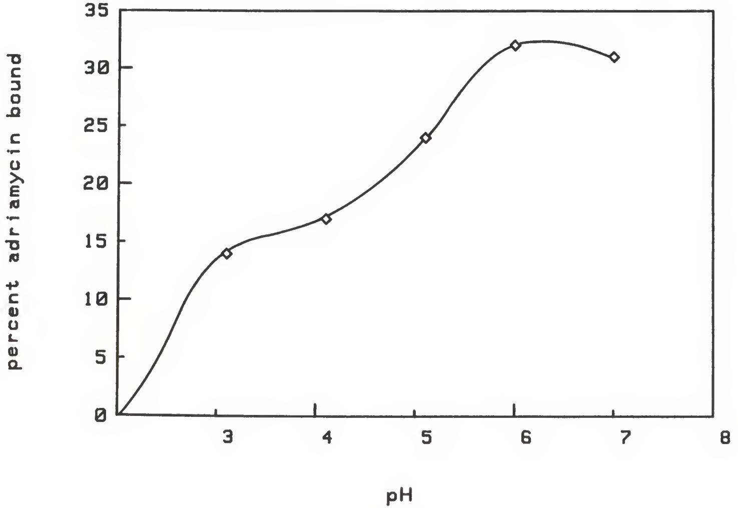


Figure 52. % AD Bound to PGA(10%)-HSA/MS-Q vs pH.

For the unquenched MS (HSA/MS-U and PGA-HSA/MS-U) with covalent AD binding, the release data is depicted in Figure 53. The amount of AD released in 15 hrs varied from 23% for AD-HSA/MS-U to 50% for AD-PGA(22%)-HSA/MS-U. Increasing the concentration of PGA in HSA/MS increased the release rate of AD. This is because the PGA-AD salt complex dissociates faster than hydrolytic degradation of covalently bound AD. Increasing the amount of incorporated PGA increases the rate of AD release. The release curves shown in Figure 54 are representative for AD-HSA/MS-Q and AD-PGA/MS-Q (3.14.5 through 3.14.8). The amount of AD released in 15 hrs varied from 55% for AD-PGA(19%)-HSA/MS-Q to over 80% for AD-HSA/MS-Q. Unlike the data shown in Figure 53, as the amount of PGA increases, the concentration of AD that is released decreases. Since the MS are quenched, there are few reactive aldehydes groups available to covalently bind AD. The predominate drug binding mechanism would therefore be physical association for HSA/MS-Q and AD-PGA salt formation for PGA-HSA/MS-Q. The physically adsorbed AD is apparently released faster than salt dissociation. Therefore, as the amount of the PGA is increased in the MS, a higher percentage of the bound AD is associated salt complex. This causes a higher concentration of AD to be released by salt dissociation with then reduces the release rate. The release data shown in Figure 55 compares 18% AD-HSA/MS that were either quenched or unquenched before addition to the AD. The top release curve

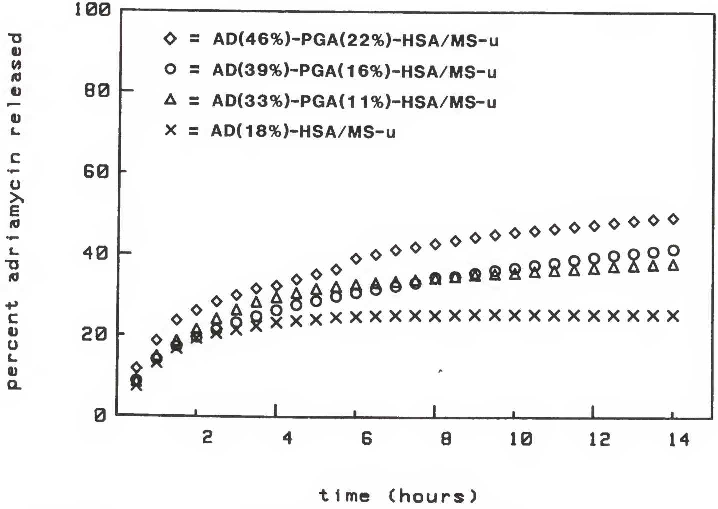


Figure 53. In Vitro Release of AD from Albumin/MS-U, Average Diameter 30 μm.

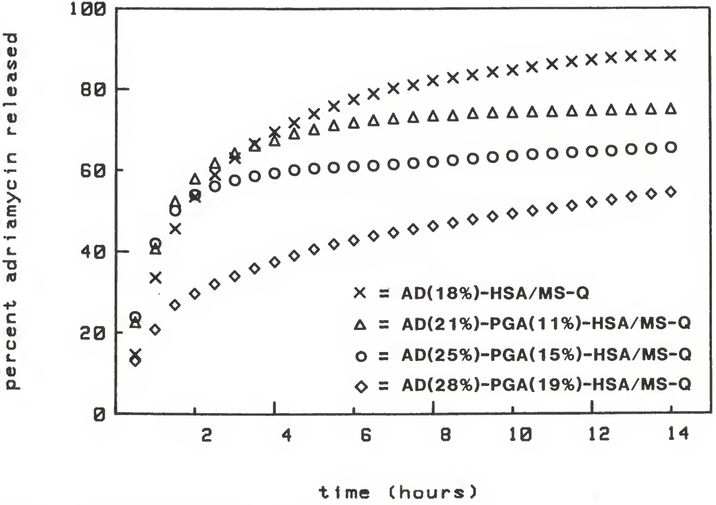


Figure 54. In Vitro Release of AD from Albumin/MS-Q, Average Diameter 26 μm.

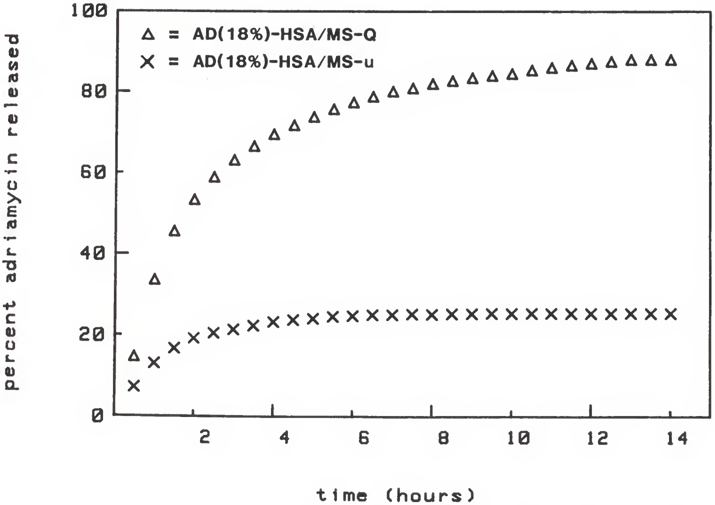


Figure 55. In Vitro Release of AD from Albumin/MS, Showing Fast and Slow Release, Average Diameter 27 μm.

in Figure 55 is for 18% AD-HSA/MS-Q. Here the predominate drug binding mechanism is by physical association and fast release is obtained. The bottom release curve represents 18% AD-HSA/MS-U, where the predominate drug binding mechanism is by covalent bonding. This gives slow release by combining physical binding (fast release) or covalent binding (slow release) with ionic salt formation, a medium range of release rates could be obtained as shown in Figure 56.

The ionic-exchange properties of the AD-PGA salt complex is further exhibited in Figure 57. For dynamic flow release of AD from AD-HSA/MS-Q (OH), AD-HSA/MS-Q (COOH) and AD-PGA-HSA/MS-Q (COOH). The OH indicates that the MS were quenched with 2-aminoethanol while COOH indicates glycine was used for the quenching process. Glycine would increase the concentration of carboxyl groups. Higher concentrations of carboxyl groups allow higher concentrations of AD binding by salt formation. Because the salt complex is stable in water, the MS with the highest concentration of carboxyl groups (AD-PGA-HSA/MS-Q, COOH) released the smallest amount of AD (4%) when water was used as the mobile elution phase. AD-HSA/MS-Q (OH), which contains the smallest amount of carboxyl groups (albumin itself contains some carboxyl groups), releases the greatest amount of AD (35%) during the water phase, Figure 57. When the water phase was replaced with saline, the AD-COOH salt dissociated releasing AD, as

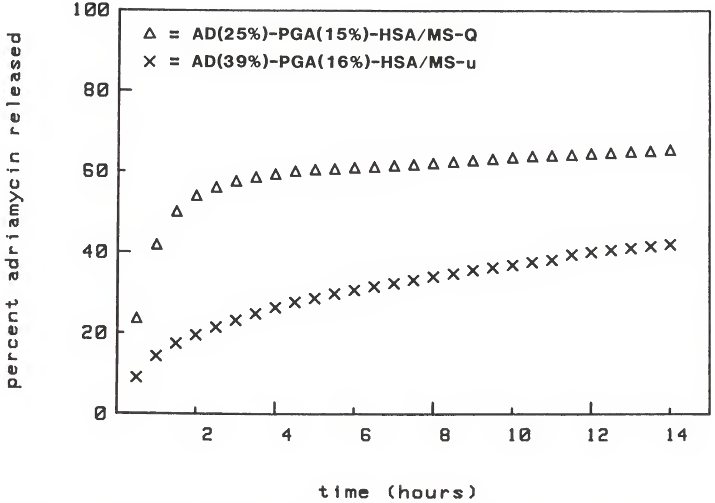


Figure 56. In Vitro Release of AD from Albumin/MS, Showing Medium (Fast) and Medium (Slow) Release, Average Diameter 29 μm.

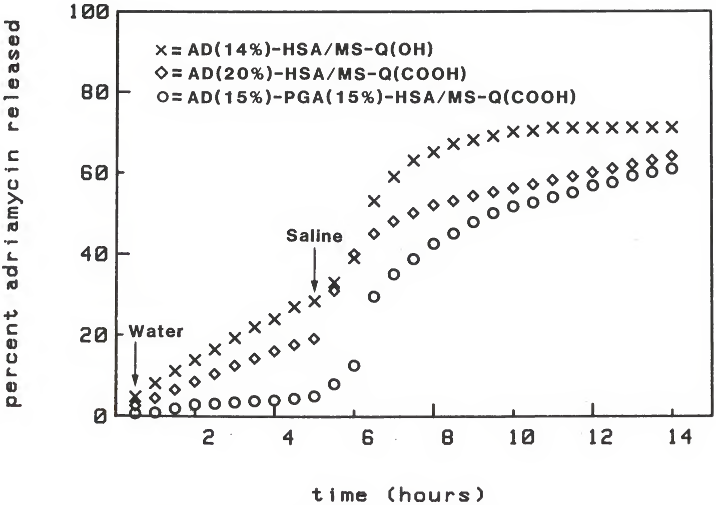


Figure 57. Ion-Exchange AD Release from AD-HSA/MS-(quenched with glycine or aminoethanol) and AD-PGA(15%)-HSA/MS-Q.

shown in Figure 57. The AD-albumin/MS used in these release experiments exhibit ion-exchange properties. This was demonstrated with AD-PGA-HSA/MS-Q (COOH) which contains a high concentration of carboxyl groups as well as AD-HSA/MS-Q (OH) containing a smaller number of COOH groups.

Dynamic flow release rates in vitro may not accurately represent kinetic behavior in vivo. Only animal studies can provide such information. However, in vitro comparisons between drug carriers are useful to guide animal testing. Furthermore, the dynamic column elution system is at best, a repesentation of drug delivery in a blood circulatory system due to the fast continuous flow. MS immobilized in solid tumors or soft tissue would not be subject to a rapid turn over of fluid. The slower fluid turnover might be better represented by a semi-static in vitro release model. In a static system, drug release from AD-HSA/MS would be regulated by the diffusional and desorption mechanisms described with a diffusional dependence on the concentration of released AD in the AD-HSA/MS environment. As the drug is released into a relatively closed system (i.e. tumor mass), an external equilibrium concentration would be reached where further drug release would be inhibited. Drug diffusion away from the tumor area would then reduce the concentration to a point where more drug would then be released from AD-HSA/MS, Figure 58 demonstrates this point. It shows the release of AD from the 33% AD-PGA-HSA/MS-Q (3.14.11) containing 26 mg of AD, suspended in 2.0 ml of saline. This

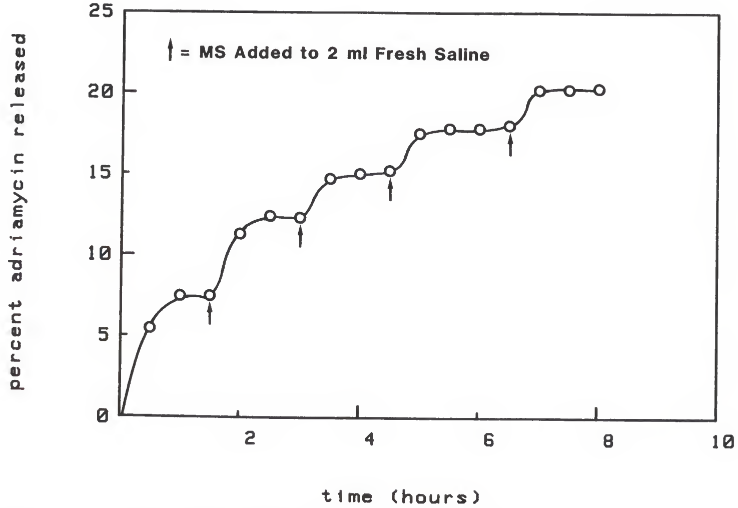


Figure 58. Fixed Volume (Static) in Vitro Release of AD from AD-PGA(12%)-HSA/MS-Q.

is equivalent to approximately 1 mg AD per ml of tumor mass volume. Figure 58 shows that the AD-PGA-HSA/MS-Q released AD until a concentration was reached where further AD release was inhibited. Once the AD-PGA-HSA/MS were removed from the saline-drug environment and added to a fresh 2 ml volume of saline, AD was again released. As the MS AD concentration was reduced, the equilibrium concentration to inhibit AD release was also reduced. HSA/MS containing therapeutic agents such as AD injected into areas that have a low fluid turnover could therefore maintain high concentrations of drug in the target area for extended periods of time.

In vivo studies. AD is an extremely toxic anti-tumor drug and must be administered in low therapeutic concentrations when used clinically. By binding AD to the MS, the toxicity of the drug may be reduced as compared to free AD. To explore this possibility, AD toxicology studies were performed using laboratory mice.

CD-1 mice were injected intraperitoneally (i.p.) with AD, 27% AD-PGA-HSA/MS and PGA-HSA/MS without drug, (3.14.12). Figure 59, show the % survival for the treated animals. A dose of 600 μg of AD killed 80% of the animals within 10 days. The same amount of AD bound to PGA-HSA/MS was not toxic in 40 days. When AD-PGA-HSA/MS with 1500 μg of bound AD was administered i.p., 60% of the mice survived until the study was terminated at 40 days. The results

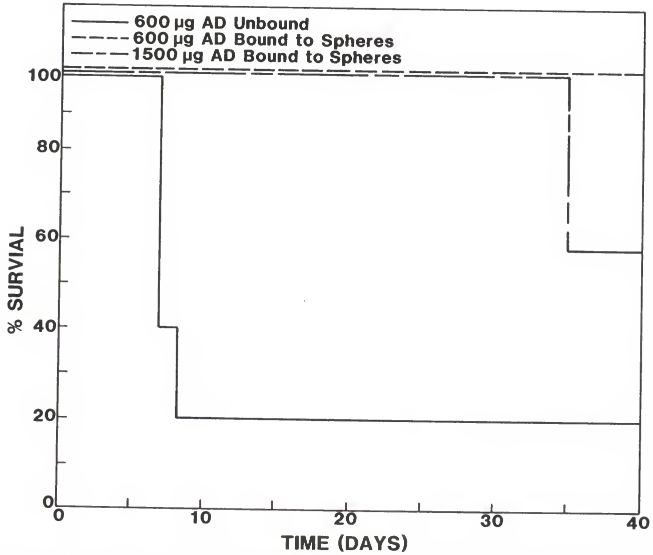


Figure 59. Toxicology Studies of AD and AD-PGA(20%)-HSA/MS-U in CD-1 Mice by i.p. Injection.

indicate that by binding AD to MS, the toxicity of the drug was greatly reduced. Thus, a much higher concentrations of AD can be used when bound to MS vs free drug.

Another clinical problem often encountered with AD is the severe necrotic lesions that develop at the site of injection when there is drug leakage around the needle. These ulcers heal very slowly and sometimes not at all. It was found that when AD (600 μg) was administered i.p., ulcer formation occurred at the injection site as shown in Figure 60. All animals treated with AD, developed these ulcers. The photograph in Figure 61 shows a mouse injected with the same dose of AD bound to MS. No ulcers formed with animals injected with AD-HSA/MS. Control group of PGA-HSA/MS showed no toxic effects.

Binding AD to HSA/MS reduced drug toxicity. Other investigators (28) have also found reduced toxicity for AD bound to large macromolecules such as dextran. The significant reduction in toxicity for AD-PGA-HSA/MS suggests that MS may be effective carriers for i.v. administration of AD as well as for localized therapy. As noted earlier however, sub-micron MS would be required for preparations of i.v. injectable compositions.

Some important properties required of insoluble MS for localized chemotherapy after injection are (1) immobilization at the injection site, (2) biodegradation, and

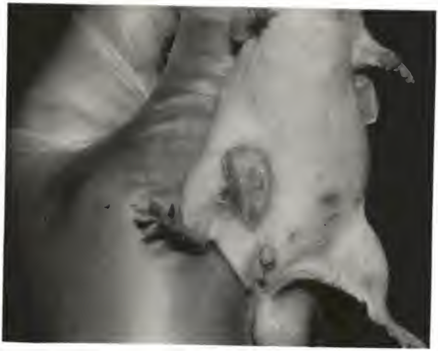


Figure 60. CD-1 Mouse Showing Ulceration from i.p. Injection of Free AD (600 μg)

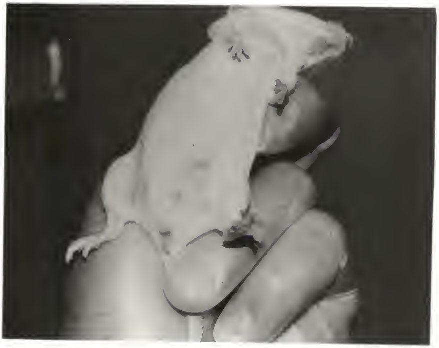


Figure 61. CD-1 Mouse Injected with AD (600 μg) Bound to PGA(20%)-HSA/MS-U, no Ulcer Formation Evident.

(3) release of the drug from the MS. Albumin/MS synthesized in this study were examined for these characteristics.

For immobilization and degradation studies, FTIC-BSA/MS (procedure 3.2.7) were injected in muscle tissue of CD-1 mice. A fluorescent optical micrograph of these MS are shown in Figure 62. At various times from 1 day to 6 weeks the muscle tissue was removed, histological slides were made and examined for MS localization and degradation. The optical micrograph shown in Figure 63 and 64 show tissue samples removed after 1 and 4 weeks MS are still located at the injection site. It was also found that at 6 weeks, the BSA/MS in this particular study were degraded. Biodegradation would be a function of cross-link density and maybe readily controlled.

AD-PGA-HSA/MS were also injected into CD-1 muscle tissue and examined histologically. AD is fluorescent and could be easily observed with the optical microscope under fluorescent illumination. Tissue samples examined four weeks after injection still showed the red chromophore of AD in the tissue surrounding the immobilized AD-HSA/MS.

This study demonstrated three important characteristics of the albumin/MS produced in this synthesis, (1) localization at the site of injection, (2) biodegradation, and (3) drug releasing properties after injection.

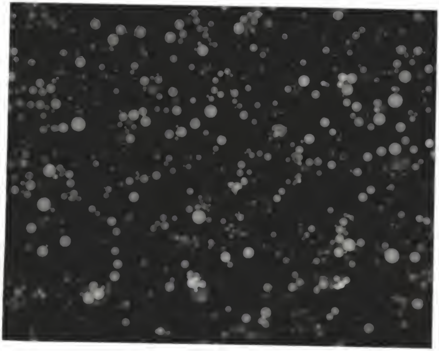


Figure 62. Optical Fluorescent Micorgraph (100 X) of BSA/MS-U that are Fluorescently Labeled, PMMA Dispersant, Average Diameter between 20-30 μm.

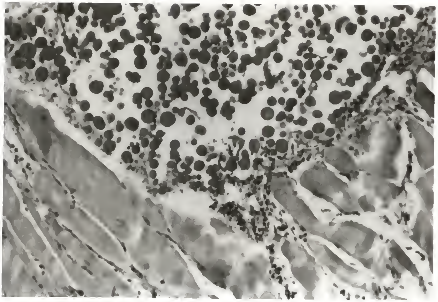


Figure 63. Optical Micrograph (100 X) of FTIC-BSA/MS-U Immobilized in Mouse Leg Muscle 1 Week After Injection.

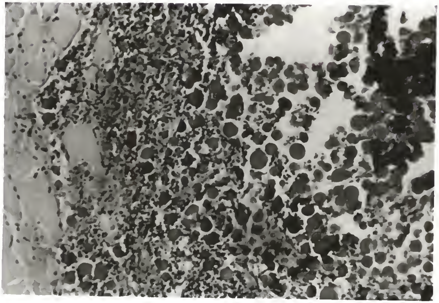


Figure 64. Optical Micrograph (100 X) of FTIC-BSA/MS-U Immobilized in Mouse Leg Muscle 4 Weeks After Injection.

4.15.2 Bleomycin-Albumin/Microspheres

BLM, another anti-tumor drug, was bound to albumin/MS at concentrations up to 30 wt %. In Table 44, the concentrations of BLM bound to HSA/MS-U was 29 wt % and to PGA-HSA/MS-U it was 30 wt % for preparations 3.15.1 through 3.15.3. The concentrations of bound BLM in HSA/MS-Q and PGA-HSA/MS-Q were 23 wt % to 30 wt % (Table 45). Because the concentration of bound drug for the two systems were similar, it was reasoned that BLM was predominately physically bound with little or no drug covalently bound to the MS. Furthermore, unlike the enhanced binding of AD to PGA-HSA/MS, increasing the concentration of PGA did not significantly increase BLM binding. These results indicate that the percentage of bound BLM to the MS was approximately equal with or without PGA. To investigate further this observation, equal molar amounts of BLM and PGA dissolved in water were combined using the technique described by Terry (38) for AD and PGA. However, unlike Terry's results with AD, BLM did not form a drug-salt complex with the PGA.

The structure of BLM is shown in Figure 50. The glycopeptide contains amino acid and sugar groups in the polycyclic structure. The amino groups should enable BLM to be chemically modified by reaction with aldehydes or by ionic salt formation with appropriate anionic macromolecules. However, all attempts to use BLM amino groups for reactions were unsuccessful. Other investigators have experienced the same difficult with the chemical

Table 44

Amount of Bound BLM for Unquenched HSA/MS and PGA-HSA-MS (Preparations 3.15.1 - 3.15.3)

Amount of Added PGA	Weight % Bound BLM
0	30
9	31
14	30

Table 45

Amount of Bound BLM for Quenched HSA/MS and PGA-HSA-MS (Preparations 3.15.4 - 3.15.6)

Amount of Added PGA	Weight % Bound AD
0	23
9	28
14	29

modification of BLM (95). Ghose et al. (96) reasoned that the molecular structure of BLM produced a sterically hindered access of reactants such as glutaraldehyde to BLM amino groups. However, BLM is sold commercially as the BLM-sulfate salt. One possible explanation for the non-reactivity of BLM is that strong binding of the sulfate ion makes displacement and reactions of the amino group difficult. The sulfate might be exchanged with a smaller acidic molecule such as HCl at an appropriate pH to produce BLM-HCl. This might react in a manner similar to AD-HCl with PGA or glutaraldehyde. Further investigation is needed to explore these possibilities.

In vitro release. The in vitro release of BLM from BLM-HSA/MS and BLM-PGA-HSA/MS was measured using the flow column described for the AD compositions and comparisons of the release of the two drugs were made.

Figures 65 and 66 show the release data for BLM-HSA/MS-U and BLM-PGA-HSA/MS-U. Figure 65 shows that by incorporating PGA (10%) into the HSA matrix, the rate of BLM release increased. Increasing the concentration of PGA from 10 to 15 wt % caused no significant change in release rate, (Figure 66). Release curves for the BLM-HSA/MS-Q and BLM-PGA-HSA/MS-Q are shown in Figures 67 and 68. Again, by incorporating PGA (10%) into the MS, the amount of BLM released increased from 45% to 55%, (Figure 67). As demonstrated with the unquenched MS, increasing the

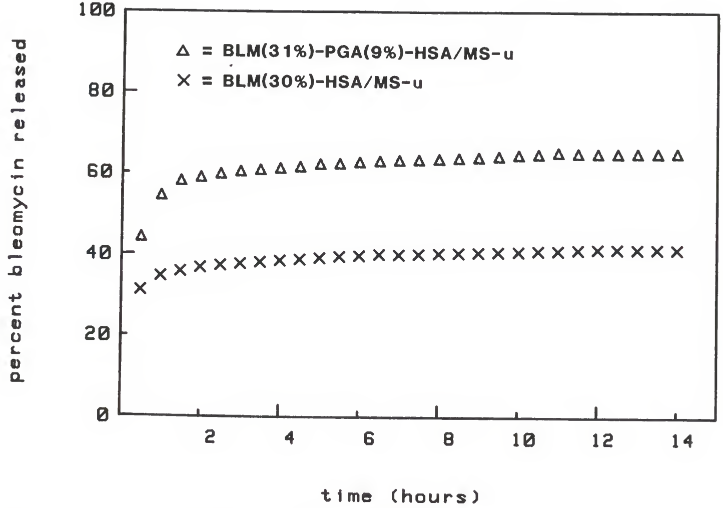


Figure 65. In Vitro Release of BLM from Albumin/MS-U, Average Diameter 29 μm.

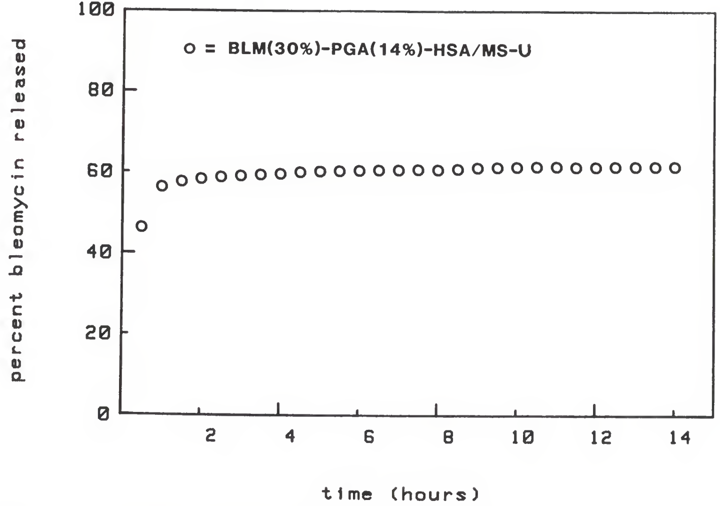


Figure 66. In Vitro Release of BLM from Albumin/MS-U, Average Diameter 31 μm.

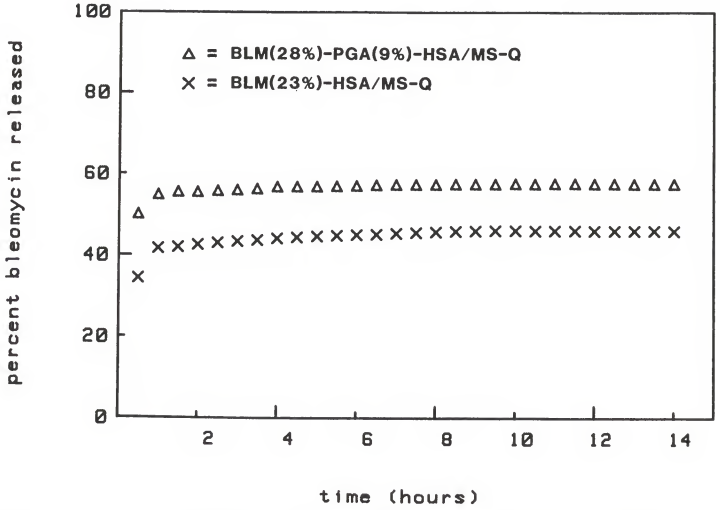


Figure 67. In Vitro Release of BLM from Albumin/MS-Q, Average Diameter 28 µm.

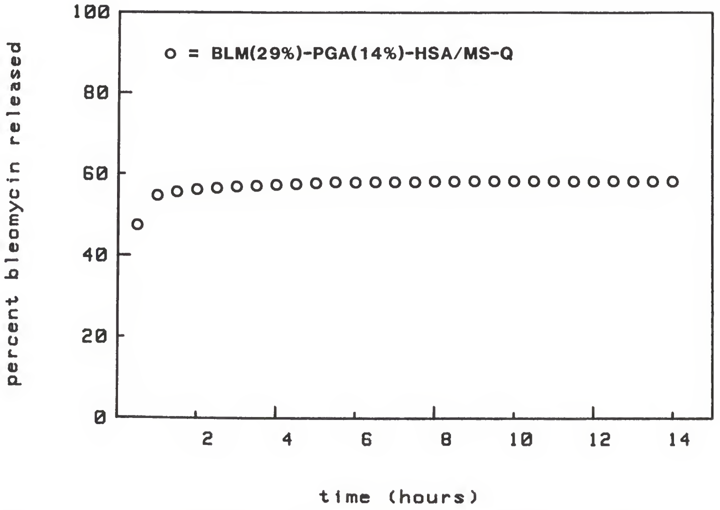


Figure 68. In Vitro Release of BLM from Albumin/MS-Q, Average Diameter 31 μm.

amount of PGA further had little effect on release rates, (Figure 68).

Since BLM-sulfate does not react with aldehydes or form ionic salts, the versatility in release rates demonstrated with AD/MS were not seen. There is however, a significant increase in release rate for BLM in vitro when PGA is combined with the HSA matrix for both the quenched and unquenched MS. This effect may be due to a reduction in cross-link density due to the incorporation of PGA. This would result in an increase in pore size of the MS, thus allowing for faster diffusion rates for BLM.

Since the BLM does not appear to bind covalently to CHO groups on unquenched MS, physical association between the glycoprotein structure of BLM and albumin is likely the dominant binding mechanism.

The in vitro data for BLM indicate two distinct release rates. A fast release within the first hour (40-55%), followed by a slow release (5-10%) for the remainder of the experiment which was to 14 hrs. The fast phase must be due to a "burst" of BLM that was on or near the surface of the BLM-HSA/MS. BLM more strongly associated with HSA would then be released more slowly. This is reflected in the second phase of the release experiment.

The use of BLM-sulfate is not reported in the drug delivery literature to any significant extent, which is probably due to the difficulty in chemically modifying BLM in such a way that it can be coupled to macromolecules with retention of biological activity. If, in the future work, BLM-sulfate can be modified to BLM-HCL then BLM uptake and subsequent release from MS might be more readily controlled.

4.15.3 Gentamycin-Albumin/Microspheres

GMC has an aminoglycoside structure and contains three primary amino groups on the sugar moities as shown in Figure 69. The drug is extremely water soluble (greater than 100 mg/ml) and basic. As a general rule the more soluble and basic a drug is the less binding affinity it has for albumin (60). This was demonstrated with the binding data for GMC and HSA/MS-U (Table 46). The MS were only able to bind 12 wt % of the antibiotic.

Table 46

Amount of Bound GMC for Unquenched HSA/MS and PGA-HSA-MS (Preparations 3.16.1 - 3.16.3)

Amount of Added PGA	Weight % Bound GMC
0	12
17	16
19	18

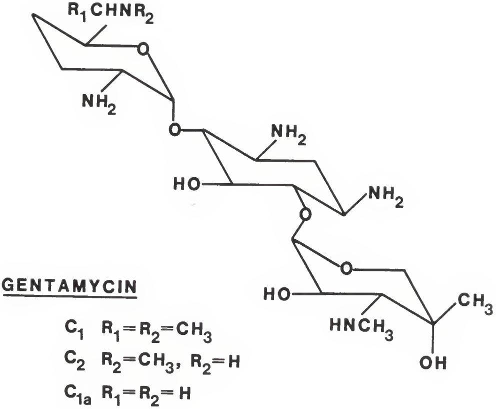


Figure 69. Structure of GMC.

However, GMC readily forms ionic salts with PGA and the addition of the anionic polymer to the albumin should greatly increase the binding concentration of GMC. However, this was not observed; Table 46 shows that incorporation of PGA into HSA/MS only slightly increased the drug concentration marginally (18 wt %).

These results may be explained by examining the structure of GMC. The drug has a low MW, 477, but is a relatively bulky molecule with 3 sugar groups in a chair configuration. The drug may not be able to effectively penetrate into the interior of the HSA/MS causing a large amount of the bound GMC to be on or near the surface of the MS. Decreasing the cross-link density of the MS may increase the pore size of the matrix so that higher concentrations of GMC may be incorporated into the MS. This requires further study.

In vitro release. The release curve for 18% GMC-HSA-PGA/MS-U is shown in Figure 70. The drug release rate was quite fast (70% in 14 hrs). Since the unquenched MS contained reactive CHO groups, a lower release rate was expected because of anticipated covalent binding of GMC. However,if the drug did not penetrate into the MS, much of the bound GMC would then be complexed or near the surface. A high percentage of the GMC on the surface would be physically bound rather than covalently bound. As previously discussed, this binding gives a fast release as shown in Figure 70.

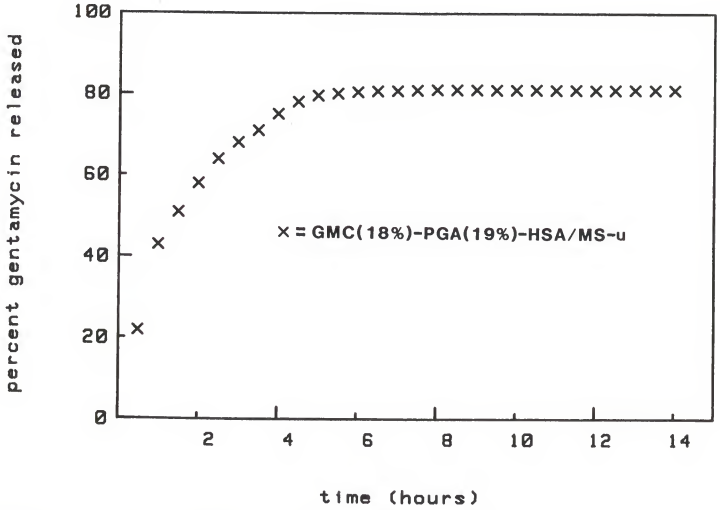
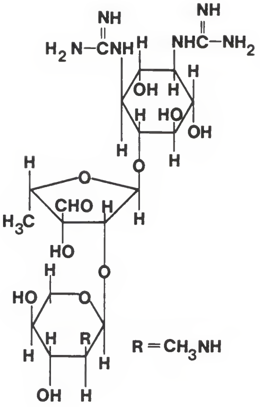


Figure 70. In Vitro Release of GMC from Albumin/MS-U, Average Diameter 17 μm.

4.15.4 Streptomycin-Albumin/Microspheres

Streptomycin (STM) is another aminoglycoside with a MW of 593 and has two primary amino groups, as shown in Figure 71. Commercially available STM is obtained as a dimer with a MW of 1456. As with AD and GMC, STM can form ionic salts with appropriate anionic polymers. The amine groups should react with glutaraldehyde by formation of a Schiff base. Thus STM was expected to bind in high concentrations to the MS. However, both HSA/MS-U and PGA-HSA/MS-U only bound 2-6 wt % of the drug, HSA/MS-Q and PGA-HSA/MS-Q bound a slightly higher concentrations of STM (4-7 wt %).

The low binding concentrations of STM indicated that the drug probably does not penetrate the interior of the MS this may again be due to the high cross-link density of the MS. When the cross-link density was reduced, PGA(14%)HSA/MS-Q to complexed 25 wt % of STM and PGA(14%)-HSA/MS-U bound 14 wt %, see Table 47. The PGA(14%)-HSA/MS-U would be expected to bind a higher concentration of STM than the quenched because of the free reactive aldehyde groups. However, if STM is covalently bound to the surface of MS-U in a sufficiently high concentration, it may inhibit other STM molecules from gaining access to the interior by a blocking effect because of the large size of STM. Quenching the aldehydes would reduce the MS surface concentration of STM, but would allow



STREPTOMYCIN

Figure 71. Structure of STM.

Table 47

Amount of Bound STM for PGA(14%)-BSA/MS (Preparations 3.17.1 and 3.17.2)

Unquenched or Quenched	Weight % Bound STM
Unquenched	25
Quenched	18

further passage of the drug into the interior of the MS, Figures 72 through 76 demonstrate this point. The optical micrographs shown in Figure 72 and 73 show BSA/MS that were dehydrated with acetone and lyophilized. The acetone-dried MS retained shape through the drying process while the lyophilized MS lost all spherical integrity. However, once rehydrated, both types of MS regained comparable geometries, Figures 74 and 75. When lyophilized BSA/MS-U were added to the STM solution (10 mg/ml), the MS swelled very little, Figure 76. Since the BSA/MS-U hydrate in water, the STM molecules must inhibit rehydration of the lyophilized BSA/MS-U due to a blocking effect of STM bound to the surface of the MS.

The physical condition of the PGA-BSA/MS had a profound effect on the amount of incorporated STM when the MS were added to the drug solution. Table 48 shows that PGA-BSA (19%)/MS-Q that was acetone dried, was able to bind the

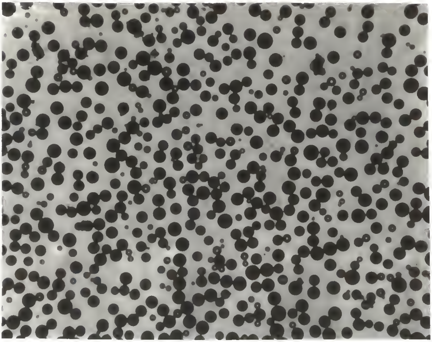


Figure 72. Optical Micrograph (100 X) of BSA/MS-U Dehydrated with Acetone.

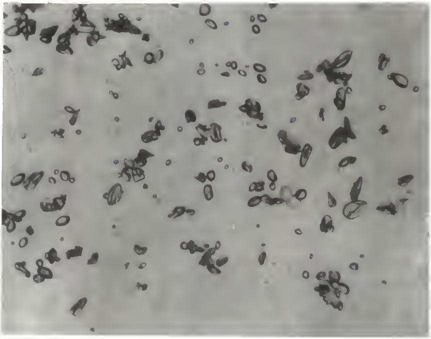


Figure 73. Optical Micrograph (100 X) of BSA/MS-U Lyophilized.

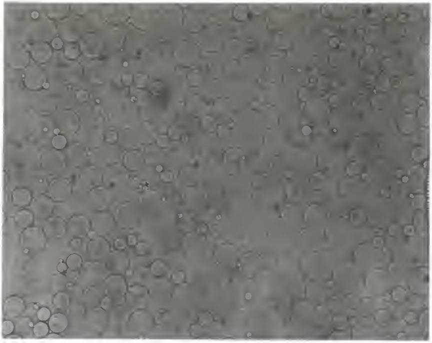


Figure 74. Optical Micrograph (100 X) of BSA/MS-U, Hydrated After Dehydration with Acetone.

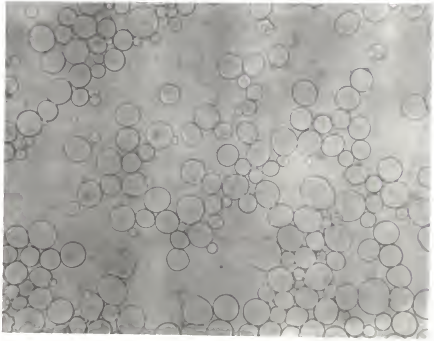


Figure 75. Optical Micrograph (100 X) of BSA/MS-U, Hydrated After Lyophilization.

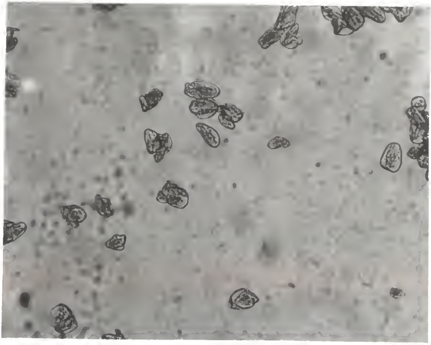


Figure 76. Optical Micrograph (100 X) of BSA/MS-U in STM Solution After Lyophilization.

Table 48

Amount of Bound STM for PGA(14%)-BSA/MS-Q
As a Function of Dehydration and Hydration Properties
(Preparation 3.17.2 - 3.17.4)

Condition of MS Upon Addition to STM Solution	Weight % Bound STM
Acetone Dried	25
Lyophilized	12
Wet	13

highest concentration to STM (25 wt %) as compared with the same MS that were either lyophilized (12 wt %) or hydrated (13 wt %). As shown, the structural changes for the lyophilized MS vs acetone dried are significant. This probably affects drug uptake. The PGA-BSA/MS-Q that were added to the STM solution in the hydrated state, also showed a low tendency for drug binding. This is probably due to the problem of STM diffusion into the PGA-BSA/MS-Q. The acetone dried PGA/BSA/MS-Q had the highest STM uptake, this result can be attributed to facilitated drug uptake during hydration with the drug solution. In vitro release data were not obtained.

The assay method for STM was not sensitive enough to determine the amount of released drug when the dynamic flow column was used. In future studies, biological assays should be tested that have the sensitivity needed for sensitive measurement of released STM.

4.16 Bovine Macrophage Uptake of Bovine Serum Albumin

Since brucellae bacteria infections in cattle reside within celluar structures (i.e. macrophage) successful treatment requires macrophage uptake of drug, e.g. injected BSA/MS containing STM. Using an activated bovine macrophage model developed by Dr. Hoffmann at the University of Florida, macrophage endocytosis of BSA/MS was studied.

Figure 77 is an optical photomicrograph of a bovine macrophage which has taken up 5 BSA/MS. The results are preliminary but suggest that BSA/MS are effectively engulfed by the activated macrophage. Further study is needed to fully explore the uptake mechanisms and to answer questions concerning endocytic uptake of STM-BSA/MS and the influence of macrophage activation and MS particle size and surface properties on MS properties on MS endocytosis.

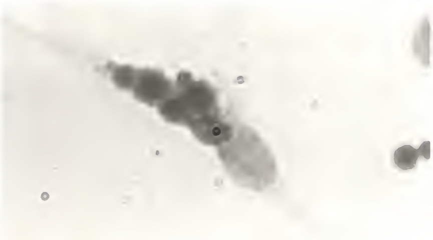


Figure 77. Optical Micrograph of (1000 X) of Bovine Macrophage Containing BSA/MS-U.

5. CONCLUSIONS

1. A new method was developed for preparing hydrophilic HSA/MS. Important aspects of this method included addition of glutaraldehyde or other cross-linking agents in the organic phase and use of concentrated polymer solutions as dispersion media. The HSA/MS were hydrophilic and easily dispersed in a variety of aqueous media without surfactants.

2. Diisocyanates were shown to be effective cross-linking agents for HSA/MS preparation. The reagents are soluble in toluene and can be introduced directly into the organic phase of the HSA dispersion.

3. Sucrose was oxidized with periodate to yield aldehyde-sucrose which was also used as a cross-linking agent for HSA/MS. Scanning electron micrographs of the aldehyde sucrose cross-linked HSA/MS were similar to MS produced with the other cross-linking reagents used in this study. The aldehyde sucrose may be a more bioacceptable alternative to glutaraldehyde or diisocyanates. It reacts more slowly than the other reagents.

4. Use of concentrated polymer solutions as the organic dispersant phase affords excellent steric stabilization of the aqueous albumin microdispersion allowing for

HSA/MS formation. The ability of the polymeric solutions to achieve stabilization was a function of polymer concentration in solution, MW, and concentration of the organic phase. As a general rule, increasing polymer concentration, MW and dispersion concentration, increased the steric stabilization of the dispersion.

5. Size distribution of HSA/MS were readily controlled from 3 to 150 μm by adjusting dispersion time and mixing energy. Sub-micro MS were also obtained by using dispersion dilution and a tissue homogenizer for mixing.

6. Cross-link density was readily controlled by adjusting the molar concentration of cross-linking agents. Cross-link density was measured by hydration swelling (size increase from the dehydrated state) and weight of water uptake.

7. The number of reactive aldehyde groups on unquenched HSA/MS (i.e. HSA/MS not treated with monofunctional amine reagents) was measured using radiolabeled leucine and concanavalin A. For 10 μm HSA/MS, 2.5×10^7 aldehyde groups were shown to covalently bind the tritiated amino acid. A small molecule like leucine was able to bind to both surface and interior aldehyde groups. However, reaction with Con-A, a large macromolecule (108,000 MW), was HSA/MS surface area dependent suggesting reaction only with free aldehyde groups on the surface of the HSA/MS.

8. Preliminary ESCA studies indicated that the polymer dispersant (PMMA), was completely removed from the HSA/MS

surface by acetone washing. The N/C ratio (in atomic percent) for the HSA/MS surface was equivalent to that measured for a cross-linked HSA sample which was not exposed to PMMA.

9. A capillary rise technique was developed to measure HSA/MS hydrophilicity. Although this procedure was not developed to the point that surface energy could be measured, it did allow quantitative comparisons of hydrophilicty for HSA/MS synthesized by different procedures. HSA/MS quenched with glycine demonstrated the highest wettability (28.6 mm capillary rise) whereas hydrophobic HSA/MS produced by the conventional vegetable oil method showed virtually no wetting (0.1 mm capillary rise).

10. HSA/MS were readily modified by incorporation of either synthetic polypeptides or chemically modified dextrans. Albumin is slightly cationic. Addition of polyglutamic acid (PGA) or carboxymethyldextran (CMD) in concentrations up to 25 wt % produced anionic HSA/MS. Alternately, incorporating polylysine (PLY) in concentrations of up to 100 wt % (pure PLY) produced MS that were more cationic in nature.

11. Serum albumin from other mammals (bovine, dog, and rabbit) were also utilized for the production of hydrophilic MS to show the versatility of the procedure.

12. When acetone dehydrated HSA/MS were mixed with the antibiotic solutions used in this study, higher

concentrations of drug were adsorbed than for HSA/MS that were either lyophilized or hydrated (swollen with water).

13. Adriamycin (AD) was bound to HSA/MS by both physical association (to 18 wt %) and covalent binding (to 18 wt %). Incorporation of PGA into the albumin increased AD binding to 45 wt % by AD-PGA salt formation. In vitro release of drug was measured for AD-HSA/MS and AD-PGA-HSA/MS using a dynamic column flow method. Three distinct release mechanisms could be achieved depending on the type of drug binding used: 1) slow--by hydrolytic degradation of covalent bonds, 2) medium--by dissociation of salt complex, and 3) fast--by release of physically adsorbed drug.

14. BLM was bound to HSA/MS and HSA-PGA/MS at concentrations up to 25-30 wt %. Unlike AD, the primary amino groups on the BLM molecule appear to be less basic, perhaps due to steric hindrance. BLM also did not readily form ionic salt complexes with polyglutamic acid. Thus, surface or bulk modification of the HSA/MS had little effect on the amount of drug complexed. The high concentration of adsorbed BLM is however attributed to strong interactions between BLM and albumin. In vitro release curves in a flow system showed two distinct processes; a fast release in the first hour due to weakly bound or surface adsorbed drug and then slow release of more tightly bound or diffusion rate limited bulk BLM.

15. The antibiotic gentamycin (GMC) demonstrated lower binding affinity for either HSA/MS (14 wt %) or HSA-PGA/MS

(18 wt %). It was shown that PGA or CMD in solution formed insoluble ionic salts with GMC, but addition of these negatively charged macromolecules into HSA/MS did not substantially increase GMC uptake. This suggests that the aminoglycoside structure was sterically too large relative to the HSA/MS pore size thus inhibiting interior penetration. In vitro release rates were also substantially faster than for other HSA/MS, indicating that a high percentage of the GMC is on or near the surface.

16. Streptomycin (STM) was incorporated into PGA-HSA/MS. By reducing the cross-link density of HSA-PGA/MS, the amount of STM was increased from 4 wt % to 25 wt %. Without PGA, HSA/MS did not show any appreciable STM binding.

17. Preliminary results indicate that bovine macrophages efficiently uptake BSA/MS and may be of use in the treatment of brucellae in cattle.

18. Immobilization of fluorescence labeled HSA/MS injected into mouse muscle tissue was studied. Optical micrographs of histological slides of muscle removed 4 weeks after implantation clearly showed HSA/MS still located at the injection site. At 6 weeks, the HSA/MS were observed to be in various stages of degradation.

HSA/MS containing AD were also injected. Muscle samples removed at 4 weeks showed the red chromophor of AD in the tissue surrounding the AD-HSA/MS, demonstrating the drug released in vivo.

19. Drugs bound to HSA/MS appear to exhibit significantly reduced toxicity. AD-HSA/MS studied in mice by i.p. injection also produced much less inflammatory reaction at the injection site as compared with free drug.

20. This new preparation method for hydrophilic HSA/MS opens up new opportunities for their chemical modification and incorporation of high concentrations of therapeutic agents, polypeptides, hormones, etc. Such HSA/MS preparations are easily injectable and biodegradable for local therapy providing tissue immobilization and controlled drug release. They are of special interest for localized chemotherapy.

6. FUTURE RESEARCH

1. Modify the polymeric dispersion procedure to reduce the average size distribution of the HSA/MS to 0.1-0.2 μm.

2. Scale up synthesis of HSA/MS (to 30-50 grams per batch).

3. Incorporate carboxymethyl cellulose into HSA/MS to enable replacement of CMD with a commercially available anionic polymer.

4. Incorporate antigens, antibodies, Con-A, protein A, and immune stimulants such as MDP into HSA/MS and test for biological activity.

5. Study labeled molecule binding to better quantitate the molecular exclusion size of the albumin matrix and drug diffusion rates for various cross-link densities.

6. Demonstrate the "case hardening" affect and gradient density of MS that may be caused by the method of cross-linking reagents used in this study.

7. Measure toxicology of MS made with the cross-linking reagents used in this study.

8. Evaluate immunology of various HSA/MS and PGA-HSA/MS compositions.

9. Investigate cross-linked density versus drug uptake for the therapeutic agents used in this study.

10. Conduct toxicity studies for BLM-MS using the CD-1 mouse model.

11. Convert BLM sulfate to BLM hydrochloride so that BLM can be chemically modified with retention of anti-tumor activity.

12. Conduct animal studies with a) tumor models, and b) bacterial and parasitic infection models to adequately test the efficacy of the HSA/MS as compared to free drugs.

13. Measure in vitro drug release under conditions which simulate static, low fluid volume tissue environments.

14. Conduct animal studies (particularly toxicology) using i.v. injection of sub-micron HSA/MS.

15. Further study capillary rise methods for surface energy of HSA/MS.

16. Conduct ESCA studies for surface analysis.

REFERENCES

(1) Capizz, R.L., Keiser, W., and Sartorelli, A.C. Seminars in Oncology, 4(2), 227 (1977).

(2) American Cancer Society, 1981 Cancer Facts and Figures, American Cancer Society, Washington, D.C. 1982.

(3) Greenwald, E.S., Cancer Chemotherapy, Medical Examination Publishing Co. Inc., Flushing, N.Y., 1977, pg. 23.

(4) Sartorelli, A.C., ed. Cancer Chemotherapy, American Chemical Society, Washington, D.C. 1976.

(5) Crooke, S.T., and Bradner, W.T. Cancer Treatment Rev., 3, 121 (1975).

(6) Morimoto, Y., Sugibyashi, K., and Kato, Y. Chem. Pharm. Bull., 29(5), 1433 (1981).

(7) Sugipayashi, K., Morimoto, Y., Nudai, T., Kato, Y., Hasegawa, A., and Takaichi, A. Chem. Pharm. Bull., 27(1), 1433 (1981).

(8) Sugipayashi, K., Morimoto, Y., Nudai, T., Kato, Y., Hasegawa, A., and Takaichi, A. Chem. Pharm. Bull., 27(1), 204 (1979).

(9) Morimoto, Y., Masayuki, A., Sugibayashi, K., Nadai, T., and Kato, K. Chem. Parm. Bull., 28(10), 3087 (1980).

(10) Tator, C.H. Cancer Chemotherapy Reports, 6, 389 (1975).

(11) Goldberg, E.P., Iwata, H., Terry, R.N., Longo, W.E., Levy, M., and Cantrell, J.I. In: Affinity Chromatography and Related Techniques, Gribnau, T.C., Visser, J., and Nivard, R.J., eds., Elsevier, Amsterdam, 1982, pg. 375.

(12) Gregoriadis, G. The Lancet 8, 241 (1981).

(13) Reference 3, pg. 100.

(14) Petrek, J.A., and Minton, J.P., Cancer, 43, 2182 (1979).

(15) Petrek, Y.A., Mavligit, G.M., Chuang, V.P., Wallace, S., Johnston, S., Benjamin, R.S., Valdivieso, M., and Hersh, E.M. Cancer, 46, 261 (1980).

(16) Tuma, R.F., Forberg, J.O., Schosser, R., and Arfors, K.E. Biblotheca. Anatomec., 18, 210 (1979).

(17) Dakhil, S., Ensminger, W., Cho, K., Niederhuber, J., Doan, K., and Wheeler, R. Cancer, 50, 631 (1982).

(18) Tuma, R.F., Forsberg, J.O., and Agerup, B. Cancer, 50, 631 (1982).

(19) Ehrlich, P. Collected Studies on Immunity, Wiley, London, 1906, Chapt. 34.

(20) Mathe, G., Tranba, L., and Berman, J.C. Acad. Sci., 246, 1626 (1959).

(21) Flecher, I. Eurp. J. Cancer, 9, 741 (1973).

(22) Guclu, A., Ghose, T., Tai, J., and Mammen, M. Eurp. J Cancer, 12, 95 (1976).

(23) Ghose, T., Blair, A.H., Vaughan, K., and Kulkarni, P. In: Drug targeting, Goldberg, E.P., ed., John Wiley and Sons, Inc., New York, 1983, pg. 1.

(24) Ghose, T., and Blair, A.H., J. Natl. Cancer Int., 61, 65 (1978).

(25) Suzuki, T., Sato, E., Goto, K., Katsurado, Y., Unno, K., and Takahasi, T. Chem. Pharm. Bull., 29(3), 844 (1981).

(26) Rowland, G.F., O'Neill, G.J., and Davis, D.A. Nature, 225, 487 (1975).

(27) Rowland, G.F. Eurp. J. Cancer, 13, 583 (1977).

(28) Arnon, R., and Hurwitz, E. In: Drug Targeting, Goldberg, E.P. ed., John Wiley and Sons, Inc., New York, 1983, pg. 23.

(29) Harding, N.G., Ann. N.Y. Acad. Sci., 186, 270 (1971).

(30) Bernstein, A., Hurwitz, E., Maron, R., Arnon R., Sela, M., and Wilchek, M. J. Natl. Cancer Inst., 60(2), 379 (1979).

(31) Bush, H., Fujiwara, E., and First, D.C. Cancer Res., 21, 371 (1961).

(32) Widder, K.J., Senyei, A.E., and Ranney, D.F. In: Advances in Pharmacology and Chemotherapy, Garrattine, S., ed., Academic Press, New York, N.Y., 1979, pg. 213.

(33) Widder, K.J., Senyei, A.E., and Sears, B. J. Pharm. Sci. USA 71(4), 379 (1982).

(34) Gregoriadis, G., and Senior, J., eds. Targeting of Drugs, Plenum Press, New York, N.Y., 1982.

(35) Ryser, H.J., and Shem, W.C., Pro. Natl. Acad. Sci. USA, 75(8), 3867 (1978).

(36) Trouet, A., Campeneere, D.D., Sencot-Malegreaux, M., and Atassi, G. Eurp. J. Cancer, 10, 405 (1974).

(37) Tokes, Z.A., Rogers, K.E., and Rembaum, A., Proc. Natl. Acad. Sci. USA, 79, 2026 (1982)

(38) Terry, R.N., M.S. Thesis, Univeristy of Florida, (1980).

(39) Scott, M.T. J. Natl. Cancer Inst., 53, 855, (1974).

(40) Reference 39, pg. 861.

(41) Likkite, V.V., and Halpern, B.N. Cancer Res., 34, 341 (1974).

(42) Talahasi, T., Ueda, S., Kono, K., and Majima, S. Cancer, 38, 1507 (1976).

(43) McLaughlin, C.A., Cantrell, J.L., Ribi, E., and Goldberg, E.P. Cancer Res, 38, 1311 (1978).

(44) Klein, E. N.Y. State J. Med., 68, 877 (1968).

(45) Nathanson, L.J. Cutaneous Path., 6 213 (1979).

(46) McLaughlin, C.A., and Goldberg, E.P. In: Targeted Drugs, Goldberg, E.P., ed., John Wiley and Sons, New York, N.Y., 1983, pg. 254.

(47) Yolles, S., Eldridge, T.L., Woodland, J.H., Blake, D.R., and Meyer, F. In: Controlled Release of Biologically Active Agents, Tanquary, A.C., and Lacey, R.E., eds., Plenum Press, New York, N.Y., 1974, pg. 177.

(48) Lee, V.H., and Robinson, J.R. In: Sustained and Controlled Release Drug Delivery Systems, Robinson, J.R., ed., Marcel Dekker, Inc., New York, N.Y., 1978, pg. 71.

(49) Folkman, J., and Long, D.M., J. Surg. Res., 4 139, (1964).

(50) Folkman, J., Long, D.M., and Rosembaum, R., Science, 154, 148 (1966).

(51) Dzuik, P.J., and Cook, B. Endocrinology, 78, 208 (1966).

(52) Kincl, F.A., Benagiano, G., and Angee, I. Steriods, 11, 673, (1968).

(53) Luzzi, L.A., J. Pharm. Sci., 59(10), 1367, (1970).

(54) Suzuki, S., Kondo, T., and Mason, S.G. Chem. Pharm. Bull., 16(8), 1629 (1968).

(55) Luzzi, L.A. Zoglio, M.A., and Maulding, H.V., J. Pharm. Sci., 59(3), 339 (1970).

(56) Yolles, S., and Morton, J.F. Acta Pharm. Svec., 15, 382 (1982)

(57) Pitt, G., Gratzi, M.M., Jeffcoat, R.A., Zweidinger, R., and Schindler, A. J. Pharm. Sci., 68(12), 1534 (1978).

(58) Koch-Weser, J., and Sellers, E.M. N. Engl. J. Med., 294, 311 (1976).

(59) Sjoholm, I., Ekman, B., Kober, A., Ljungstedt, P., Seviving, B., and Sjodin, T. Mol. Pharm., 16, 767 (1979).

(60) Reference 58, pg. 526.

(61) Yapel, A.F. U.S. Patent 4,147,767 (1978).

(62) Kramer, P.A. J. Pharm. Sci., 63, 1646 (1974).

(63) Senyei, A.E., Widder, K.J. U.S. Patent 4,357,259 (1982).

(64) Royer, G.P. U.S. Patent 4,349,530 (1982).

(65) Wakiyama, N., Juni, K., and Nakano, M. Chem. Pharm. Bull., 30(7), 2621 (1982).

(66) Wakyama, N., Kazuhiko, J., and Nakano, M. Chem. Pharm. Bull., 30(10), 3719 (1982).

(67) Widder, K.J., Senyei, A.E., and Ranney, D.F. Cancer Res., 40, 3512 (1980).

(68) Mosback, K., Schroder, U. FEBS Letters, 102(1), 112 (1979).

(69) Senyi, A., Widder, K., and Gzerlinski, G.J. Appel. Phys., 49, 3578 (1978).

(70) Widder, K.J., Morris, R.M., Poore, G., Howard, D.P., and Senyi, A.E. Proc. Natl. Acad. Sci. USA, 78(1), 579 (1981).

(71) Sugibayashi, K., Okumura, M., and Morimoto, Y., Biomaterials, 3, 181 (1982).

(72) Kramer, P.A., and Burnstein, T. Life Science, 19, 515 (1976).

(73) Ishizaka, T., Endo, K., and Koish, M. J. Pharm. Sci. 70(4), 358 (1981).

(74) Lee, T.K., Sokoloski, T.D., and Royer, G.P. Science, 213, 4503 (1982).

(75) Rhodes, B.A., Zolle, I., Buchanan, J.W., and Wagner, H.N. Radiology, 92, 1453 (1969).

(76) Senyi, A.E., Reich, S.D., Gonczy, C., and Widder, K.J. J. Pharm. Sci., 70, 328 (1981).

(77) Widder, K.J., Flouret, G., and Senyei, A.E. J. Pharm. Sci., 68, 79 (1979).

(78) Laibson, P.R. Arch. Ophthalmol., 88, 553 (1972).

(79) Davis, S.D., Sarft, L.D., and Hydiuk, R.A. Arch. Opthalmol., 95, 1638 (1977).

(80) Sloan, S.H., Pettit, T.H., and Litwack, K.D. Am. J. Opthalmol., 73, 750 (1972).

(81) Neter, E. Medical Microbiology, F.A. Davis Co., Philadelphia, 1966.

(82) Robbins, S.L. Textbook of Pathology with Clinical Applications, W.B. Saunders Co., Philadelphia, 1966.

(83) Silva, M., Hazleman, B.L., Page Thomas, D.P., and Wraight, P. The Lancet, 6 1320 (1979).

(84) Pitha, J., Zawadzki, S., Chytll, F., Lotan, D., and Lotan, R. J. Natl. Cancer Inst., 65, 5 (1980).

(85) Windholz, M., Budavari, S., Stroumtsos, L.Y., and Fertig, M.N. The Merck Index, Merck & Co., Inc., Rahway, N.J., 1976, pg. 171.

(86) Barends, D.M., Zwaan, C.L., and Hulschuff, A. J. Chromatogr, 222, 316 (1981).

(87) Grove, D.C., and Randall, W.A. In: Assay Methods of Antibiotics a Laboratory Manual, Welch, H., and Marti-Ibaneze, F., eds., Medical Encyclopedia, Inc., New York, N.Y., 1975, pg. 34.

(88) Arnold, J.A., Nelson, J.A., and Verbanc, J.J. J. Chem. Educ., 31, 188 (1954).

(89) Heller, W. Pure Appl. Chem., 12, 249 (1966).

(90) Rswick, E., Hauser, T.R., Stanley, T.W., and Elbert, W. Anal. Chem., 33, 93 (1961).

(91) Ikada, Y., Iwata, H., Mita, T., and Nagaoka, S. J. Biomed. Mater. Res., 13 607 (1976).

(92) Richards, T.W., and Carver, E.K. J. Am. Chem. Soc. 43, 827 (1921).

(93) Dalla Valle, J.M. Micrometritics, The Technology of Fine Particles, Pitman Publishing Co., New York, N.Y., 1954, pg. 234.

(94) Ryser, H.J., Shen, W.C., and Merk, F.B. Life Sci., 22, 1253 (1978).

(95) Failla, D., and Santi, D.V. Anal. Biochem., 52, 363 (1973).

(96) Reference 23, pg. 18.

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